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TITOLO TESI

Valutazione della qualità ed autenticità di prodotti oleari e lattiero caseari mediante metodi fisici di analisi accoppiati a tecniche statistiche multivariate

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1. Sommario

Oggi si usa sempre più spesso il suffisso omico per designare l'applicazione in diversi campi scientifici dell'approccio fruttuoso della metabolomica. La metabolomica è lo studio sistematico delle uniche tracce chimiche lasciate da specifici processi metabolici. Il primo vero lavoro scientifico che ha visto l'applicazione della metabolomica risale al 1971 (Pauling, Robinson, Teranishi, Cary, 1971). L'idea concepita da Pauling e collaboratori era che i dati ricchi di informazioni che riflettono lo stato funzionale di un sistema biologico complesso risiede nel modello quantitativo e qualitativo dei metaboliti nei fluidi corporei. Vent'anni dopo questo approccio fu ampiamente applicato per via della più diffusa disponibilità degli strumenti necessari a analizzare tali informazioni. Sicuramente il successo delle scienze omiche è stato favorito dalla disponibilità di strumenti statistici più rapidi e di più facile utilizzo. Partendo da queste premesse ed utilizzando strumenti analitici diversi da quelli utilizzati normalmente nelle scienze omiche (tecniche separative e spettrometriche e risonanza magnetica nucleare) in questo lavoro di tesi sono stati combinati metodi fisici di analisi accoppiati a tecniche statistiche multivariate per valutare tanto la qualità così come l'autenticità di alcuni prodotti alimentari (in particolare prodotti oleari e lattiero caseari).

L'applicazione di strumenti fisici permette di abbattere i costi ed i tempi necessari per le analisi classiche ed allo stesso tempo può fornire un insieme diverso di informazioni che possono riguardare tanto la qualità come l'autenticità di prodotti.

Per il buon funzionamento di tali metodi è necessaria la costruzione di modelli statistici robusti che utilizzino set di dati correttamente raccolti e rappresentativi del campo di applicazione.

In questo lavoro di tesi sono stati analizzati oli vegetali e alcune tipologie di formaggi (in particolare pecorini per due lavori di ricerca e Parmigiano-Reggiano per un altro).

Sono stati utilizzati diversi strumenti di analisi (metodi fisici), in particolare la spettroscopia, l'analisi termica differenziale, il naso elettronico, oltre a metodiche separative tradizionali.

I dati ottenuti dalle analisi sono stati trattati mediante diverse tecniche statistiche, soprattutto: minimi quadrati parziali; regressione lineare multipla ed analisi discriminante lineare.

Keywords: Olio Vegetale; Olio vergine di oliva; Formaggio Pecorino; Parmigiano-Reggiano; Analisi termica differenziale; DSC; Spettroscopia; FT-IR; FT-NIR; Naso elettronico; Analisi discriminante lineare; LDA; Minimi quadrati parziali; PLS; Regressione lineare multipla; MLR; Analisi dei componenti principali.

Indice

3. Lista delle Abbreviazioni

ANN	Rete neurale artificiale
ATR	Riflettanza totale attenuata
CE	Comunità Europea
CGC	Gas cromatografia capillare
DSC	Analisi termica differenziale
FT-IR	Spettroscopia infrarossa a trasformata di Fourier
GC	Gas cromatografia
GC-MS	Gas cromatografo accoppiato a spettrometro di massa
HPLC	Cromatografia liquida ad alta prestazione
IRE	Elemento interno di riflettanza
LDA	Analisi discriminante lineare
MIR	Spettroscopia nel medio infrarosso
MLR	Regressione lineare multipla
MOS	Ossidi metallici semiconduttori
NIR	Spettroscopia nel vicino infrarosso
OFA	Acidi grassi ossidati
PCA	Analisi delle componenti principali
PLS	Minimi quadrati parziali
TLC	Cromatografia su strato sottile
TMS	Trimetilsilil derivati
VOO	Olio vergine di oliva

4. Struttura della tesi

Questa tesi si basa sui lavori sperimentali condotti durante il triennio di dottorato che hanno originato 13 diversi manoscritti pubblicati durante questo periodo su alcune riviste scientifiche la gran parte delle quali sono classificate dall'Institute for Scientific Information (ISI). Pertanto la presente tesi riporta un'introduzione generale che prende in considerazione le due tipologie di matrici oggetto del lavoro di tesi (oli vegetali e formaggi), oltre ai principali strumenti analitici utilizzati durante le attività sperimentali nonché una breve illustrazione degli strumenti di analisi statistica multivariata. Per le specifiche disamine dello stato dell'arte, nonché per le specifiche sezioni di materiali e metodi e risultati e discussioni di ciascuna attività sperimentale si rimanda ad ognuno degli articoli pubblicati. Nel teso tali articoli sono menzionati come **articolo 1-13** e di seguito sono elencati in ordine cronologico di pubblicazione (IF complessivo pari a 35,738):

Articolo 1 Lerma-García, M.J., Gori, A., **Cerretani, L.**, Simó-Alfonso, E.F., Caboni, M.F. Classification of Pecorino cheeses produced in Italy according to their ripening time and manufacturing technique using Fourier transform infrared spectroscopy. *Journal of Dairy Science* 93 (10), pp. 4490-4496, 2010 (IF 2,564).

Articolo 2 **Cerretani, L.**, Giuliani, A., Maggio, R.M., Bendini, A., Toschi, T.G., Cichelli, A. Rapid FTIR determination of water, phenolics and antioxidant activity of olive oil. *European Journal of Lipid Science and Technology* 112 (10), pp. 1150-1157, 2010 (IF 1,733).

Articolo 3 Lerma-García, M.J., **Cerretani, L.**, Cevoli, C., Simó-Alfonso, E.F., Bendini, A., Toschi, T.G. Use of electronic nose to determine defect percentage in oils. Comparison with sensory panel results. *Sensors and Actuators, B: Chemical* 147 (1), pp. 283-289, 2010 (IF 3,898).

Articolo 4 Maggio, R.M., Valli, E., Bendini, A., Gómez-Caravaca, A.M., Toschi, T.G., **Cerretani, L.** A spectroscopic and chemometric study of virgin olive oils subjected to thermal stress. *Food Chemistry* 127 (1), pp. 216-221, 2011 (IF 3,655).

Articolo 5 **Cerretani, L.**, Maggio, R.M., Barnaba, C., Toschi, T.G., Chiavaro, E. Application of partial least square regression to differential scanning calorimetry data for fatty acid quantitation in olive oil. *Food Chemistry* 127 (4), pp. 1899-1904, 2011 (IF 3,655).

Articolo 6 Cevoli, C., **Cerretani, L.**, Gori, A., Caboni, M.F., Gallina Toschi, T., Fabbri, A. Classification of Pecorino cheeses using electronic nose combined with artificial neural network and comparison with GC-MS analysis of volatile compounds. *Food Chemistry* 129 (3), pp. 1315-1319, 2011 (IF 3,655).

Articolo 7 Lerma-García, M.J., Simó-Alfonso, E.F., Bendini, A., **Cerretani, L.** Rapid evaluation of oxidised fatty acid concentration in virgin olive oil using Fourier-

transform infrared spectroscopy and multiple linear regression. *Food Chemistry* 124 (2), pp. 679-684, 2011 (IF 3,655).

Articolo 8 Lerma-García, M.J., Lusardi, R., Chiavaro, E., **Cerretani, L.**, Bendini, A., Ramis-Ramos, G., Simó-Alfonso, E.F. Use of triacylglycerol profiles established by high performance liquid chromatography with ultraviolet-visible detection to predict the botanical origin of vegetable oils. *Journal of Chromatography A* 1218 (42), pp. 7521-7527, 2011 (IF 4,531).

Articolo 9 Maggio, R.M., **Cerretani, L.**, Barnaba, C., Chiavaro, E. Application of differential scanning calorimetry-chemometric coupled procedure to the evaluation of thermo-oxidation on extra virgin olive oil. *Food Biophysics* 7 (2), pp. 114-123, 2012 (IF 2,187).

Articolo 10 Gori, A., Maggio, R.M., **Cerretani, L.**, Nocetti, M., Caboni, M.F. Discrimination of grated cheeses by Fourier transform infrared spectroscopy coupled with chemometric techniques. *International Dairy Journal* 23 (2), pp. 115-120, 2012 (IF 2,401).

Articolo 11 Bendini, A., Valli, E., Rocculi, P., Romani, S., **Cerretani, L.**, Gallina Toschi, T. A new patented system to filter cloudy extra virgin olive oil. *Current Nutrition & Food Science* 9, pp. 43-51, 2013.

Articolo 12 Valli, E., Bendini, A., Maggio, R.M., **Cerretani, L.**, Toschi, T.G., Casiraghi, E., Lercker, G. Detection of low-quality extra virgin olive oils by fatty acid alkyl esters evaluation: A preliminary and fast mid-infrared spectroscopy discrimination by a chemometric approach. *International Journal of Food Science and Technology* 48 (3), pp. 548-555, 2013 (IF 1,259).

Articolo 13 Gómez-Caravaca, A.M., Maggio, R.M., Verardo, V., Cichelli, A., **Cerretani, L.** Fourier transform infrared spectroscopy-Partial Least Squares (FTIR-PLS) coupled procedure application for the evaluation of fly attack on olive oil quality. *LWT - Food Science and Technology* 50 (1), pp. 153-159, 2013 (IF 2,545).

5. Introduzione

5.1 Le analisi per la valutazione della qualità e genuinità degli oli e dei formaggi

Il concetto di qualità è sicuramente complesso ed ampio, infatti riguarda ambiti diversi per il consumatore che contribuiscono con pesi differenti in funzione dello specifico prodotto alimentare. In prima approssimazione sono quattro gli aspetti principali: la qualità igienica, la qualità nutrizionale, la qualità edonistica e la qualità socio-culturale [1].

La **qualità igienica** o la sicurezza di un prodotto alimentare è al primo posto nelle scelte di tutti i parametri della produzione agro-alimentare: tuttavia, come è immediato ricordare a causa dei recenti scandali verificatisi per alcuni tipi di carne, di prodotti vegetali o in passato per il vino, non può essere considerata scontata, come invece dovrebbe essere. Il settore dei prodotti farmaceutici ha una regolamentazione molto più esigente di quella dei prodotti alimentari, anche se per molti casi gli alimenti possono essere veicolo di molte sostanze attive e assomigliare nei meccanismi di azione e nel sistema di introduzione, nel bene o nel male, a veri e propri farmaci.

La **qualità nutrizionale** di un prodotto alimentare viene spesso resa nota attraverso gli strumenti di comunicazioni a disposizione delle aziende produttrici nel rispetto della normativa vigente che negli ultimi anni è stata rivista con l'obiettivo di tutelare maggiormente il consumatore [2]. Infatti, una buona educazione alimentare, condotta fin dalle scuole primarie così come la corretta informazione possono trainare il consumatore verso una maggiore consapevolezza all'acquisto e verso una maggiore attenzione agli aspetti nutrizionale. Sempre in questa direzione, va sottolineato che recentemente la legislazione relativa alle indicazioni nutrizionali e salutistiche sulle indicazioni che possono essere poste nelle etichette dei prodotti alimentari [3] ha inserito le dovute restrizioni, utili allo scopo di normare i messaggi.

La **qualità edonistica** di un prodotto alimentare ha forte presa sul consumatore e, purtroppo, è spesso trainante negli acquisti e quindi è obiettivo primario della produzione di alimenti, più di ogni altro aspetto di qualità. L'accettazione di un prodotto alimentare, condizionata da alcuni fattori dei quali l'aspetto edonistico è il principale, è ritenuta dalle aziende produttive più importante di qualsiasi altro parametro per le scelte di produzione. Si acquista molto di più quello che piace, senza far considerazioni di altro tipo e, anche in questo caso, l'educazione alimentare rappresenta un elemento importante.

La **qualità socio-culturale** è quella che lega il consumatore alle sue tradizioni e alle sue origini, che sono fortissime anche in fatto di alimenti. E' ben noto come sia importante per l'uomo il legame con il paese di origine, ravvivato spesso solo attraverso il rito del cibo: certe preparazioni o determinati piatti vengono "religiosamente" tramandati dagli emigrati ai loro figli nelle nuove nazioni, così come gli immigrati in un determinato paese fanno fiorire le imprese che producono i loro cibi tradizionali. I prodotti "tipici" sono la definizione della tradizione e la loro elevata accettazione è il segno di un certo piacere del consumatore nel ritorno al passato, come legame con la tradizione. E' anche qualità socio-culturale quella che viene di solito attribuita al cibo consumato in piacevole compagnia, che per questo appare migliore di quello che realmente sarebbe in un consumo solitario. I mutamenti degli stili di vita e dei sistemi di approvvigionamento dei prodotti alimentari hanno condizionato anche i sistemi di produzione delle materie prime e, pertanto, è necessario un certo spirito di adattamento nei settori della produzione primaria [1].
Nell'ambito di questi cambiamenti, la produzione primaria si trova a scegliere fra prodotti destinati alla grande industria di trasformazione (come ad es. le colture da estrazione, come oleaginose, barbabietole da zucchero, mais da amideria, ecc.) e produzioni particolari, con vendita diretta o con vendita per settori di "nicchia" (piante officinali, oli di oliva di pregio, insaccati, formaggi, vini di pregio, ecc.) [1].

5.1.1 La qualità degli oli alimentari

Gli oli ed i grassi alimentari sono deteriorabili e possono essere ottenuti da fonti naturali, che non sempre si trovano in condizioni di conservazione e di qualità ottimali. In particolare, si possono osservare oli e grassi ossidati fin dal momento di estrazione dalla matrice dalla quale si ottengono, per cui vengono sottoposti a processi di raffinazione in modo da essere resi commestibili. La raffinazione delle sostanze grasse opera una concreta eliminazione di diverse sostanze ossidate, soprattutto quelle polari e quelle volatili, ma non riesce ad evitare una parte residua ossidata che, permanendo nel prodotto a fine lavorazione, ne determinerà il successivo tempo di conservazione (shelf life). Oltre a ciò, l'eliminazione delle sostanze polari o volatili, impoverisce la sostanza grassa dei suoi importanti componenti minori, interessanti per gli aspetti salutistico-nutrizionali, tra i quali sono spesso presenti anche dei composti antiossidanti. Pertanto, la conservabilità (serbevolezza) è un parametro di qualità di una sostanza grassa, a parità di tutti gli eventuali parametri di qualità associati ai prodotti alimentari.

Un altro importante parametro qualitativo si riferisce alle caratteristiche sensoriali delle sostanze grasse, questo aspetto è particolarmente importante nel caso degli oli provenienti dalla lavorazione delle olive, infatti tutti gli altri oli sono per regola da raffinare e, pertanto, perdono a seguito di questi trattamenti i composti responsabili delle caratteristiche sensorialmente riscontrabili [1].

5.1.2 Il caso degli oli ottenuti dalla lavorazione delle olive

Gli oli provenienti dalla lavorazione delle olive rappresentano uno dei più antichi alimenti. Insieme all'albero di olivo essi sono infatti noti da millenni e, anche se nel tempo hanno avuto qualche altro impiego oltre a quello alimentare, ad esempio come unguento per gli atleti, come fonte di illuminazione e di riscaldamento, oggi hanno praticamente solo la destinazione a condimento o come mezzo di cottura.

Gli oli provenienti dalla lavorazione delle olive sono apprezzati da numerosissimi consumatori. Tuttavia, se si chiede a diverse persone di assaggiare oli di provenienza differente, la preferenza cade tendenzialmente su uno solo ovvero quello più simile agli oli abitualmente consumati e/o prodotti nelle loro zone di origine. In altre parole, il legame fra l'abitudine alimentare della regione o della zona d'origine del consumatore e la valutazione soggettiva della qualità è molto forte, più che per molti altri aspetti tradizionali.

Le caratteristiche degli oli prodotti nelle numerosissime zone vocate alla olivicoltura sono condizionate da molteplici fattori, primo dei quali è il sistema di abitudini colturali e di quelle legate alla lavorazione delle olive raccolte (Schema 1). Si consumano preferibilmente, quindi, oli che sono prodotti secondo la tradizione delle proprie zone di origine, caratteristica questa che risulta molto variabile in un paese come l'Italia, che presenta una grande frammentazione nella produzione olivicola, oltre che un'elevata variabilità delle condizioni ambientali e pedo-climatiche [1].

Schema 1
Filiera produttiva degli oli d'oliva, in relazione alla formazione delle caratteristiche di qualità degli oli al consumo

Nel caso degli oli d'oliva, il concetto di qualità è poco definibile sulla base del gusto che, per le ragioni esposte, è particolarmente variabile. Allora, per definire le caratteristiche di qualità degli oli vergini è necessario rifarsi ad altri parametri di valutazione.

Se consideriamo l'olio ottenuto dalle olive come una "conserva" di olive, così come il formaggio lo è del latte, i salumi della carne e, forse, il vino dell'uva, le confetture dei diversi tipi di frutta e via dicendo, allora ci si aspetta che l'olio ricordi le caratteristiche della materia prima oliva. Essendo numerose le cultivar di olive con caratteristiche organolettiche molto differenti, in corrispondenza vi saranno molti tipi di oli diversi pur tutti potenzialmente di buona qualità. Oltre a ciò, l'olio dovrebbe mantenere queste caratteristiche per periodi prolungati, almeno un anno, in relazione al genere di produzione che è di tipo annuale.

Negli ultimi decenni si sono verificati profondi cambiamenti nella conoscenza degli aspetti nutrizionali dell'olio di oliva, tanto da considerarlo alla base della cosiddetta "dieta mediterranea" e quindi ricco di importanti principi nutrizionali indispensabili, quali gli

acidi grassi monoinsaturi e gli acidi grassi essenziali e di particolari componenti della dieta utili alla conservazione nel tempo delle caratteristiche citate e, forse, anche al mantenimento della salute, quali gli antiossidanti. Rispetto a tutti gli altri oli, gli oli vergini d'oliva possiedono marcate proprietà organolettiche, carenti o addirittura assenti nelle altre sostanze grasse, a causa delle pratiche tecnologiche di produzione. In virtù di ciò sono sempre più apprezzati in cucina per la loro capacità di rendere più appetitosi molti cibi e pertanto risultano più diffusi nei consumi.

Per tradizione culturale e per la particolare struttura del territorio italiano, le zone di produzione delle olive e la localizzazione dei corrispondenti frantoi sono molto frammentate, portando di conseguenza ad una miriade di prodotti differenti, seppure molti di elevata qualità individuale.

Le caratteristiche riconducibili alle singole cultivar che danno luogo alle differenze sensoriali degli oli di diversa provenienza sono riscontrabili anche dai consumatori in relazione ai propri gusti, ma molto spesso non è considerato un pregio nelle produzioni a carattere industriale bensì un difetto. Infatti, la costanza delle caratteristiche di un prodotto, anche per quelli di tipo non alimentare, è la prima prerogativa che deve possedere per essere commercializzato: con proprietà ottime, buone o mediocri, spesso non importa, basta che sia costante. Naturalmente, nel caso di agro-alimenti, è più facile garantire negli anni la costanza di una qualità mediocre piuttosto che quella di una qualità ottimale, spesso dipendente dagli andamenti climatici, per cui la tendenza è quella di orientarsi verso la mediocrità, facendo miscele in modo da rendere il più possibile uguale e costante il prodotto. Tutto ciò è facilitato da un largo consumo che mediamente rende il consumatore non in grado di distinguere le differenze organolettiche.

Produrre bene, oggi, non è sufficiente e, in generale, ha costi di lavorazione superiori alle produzioni meno curate, per cui si evidenzia, salvo eccezioni, una tendenza verso le produzioni meno qualificate e meno onerose. Tuttavia i consumatori attenti possono rivolgere le proprie attenzioni verso i prodotti tipici che hanno caratteristiche peculiari. Questa tendenza ha portato alla nascita di mercati di nicchia, come sono quelli delle denominazioni di origine o degli oli monovarietali, che garantiscono la provenienza territoriale oltre alla presenza di specifiche caratteristiche di pregio. Per quanto riguarda quest'ultimo settore, si può facilmente comprendere l'utilità del mantenimento di tali differenze assaggiando da profani oli monovarietali diversi: ad esempio quello prodotto dalla varietà siciliana Tonda Iblea caratterizzata da profumi che ricordano il pomodoro, o la pugliese Cellina di Nardò con evidenti note riconducibili al frutto del lampone, piuttosto

che il monovarietale romagnolo prodotto dalle olive Ghiacciola caratterizzato da odori erbacei che rimandano al carciofo [1].

5.1.3 Come misurare o controllare obiettivamente la qualità degli oli d'oliva

In generale le condizioni sono delineate nel regolamento della CE [4], sulle caratteristiche degli oli provenienti dalla lavorazione delle olive e sul loro controllo. Infatti, stabilito che l'olio non debba contenere sostanze estranee alle olive (come ad es. gli idrocarburi alogenati), che non debba essere ottenuto con miscelazione o mezzi illeciti, non deve presentare livelli di difetti al di sopra di una certa entità (panel test).

Chi produce oli direttamente dalle olive sa che i controlli che dovrebbe condurre sono piuttosto limitati: oltre alle caratteristiche organolettiche, sono da verificare l'acidità, gli alchil esteri ed il valore dei perossidi (numero di perossidi, NP), poiché ancora oggi non si è in grado di tenere sempre sotto controllo le variabili che influenzano questi due parametri attraverso altre scelte ed è necessario classificare su tali valori la categoria commerciale, oltre all'analisi organolettica, per rilevare e valutare eventuali difetti. Tutte le altre analisi previste non è necessario svolgerle, in questo caso. Chi è costretto, invece, a controllare a fondo l'olio, oltre alle strutture ufficiali preposte allo scopo, è chi l'acquista e lo imbottiglia, e/o lo commercializza, per assicurarsi della corretta dichiarazione posta o da porre in etichetta [1].

In sostanza, non è problematico, al livello della produzione primaria il controllo "commerciale" dell'olio; il problema semmai è come meglio produrlo per ridurre al minimo i parametri: acidità, alchil esteri, numero di perossidi e caratteristiche sensoriali.

5.1.4 La presenza e i ruolo dell'acqua negli oli vergini di oliva

L'acqua negli alimenti esercita un comportamento differente, in funzione della quantità presente, sull'ossidazione dei lipidi: a basse concentrazioni non può opporsi all'ossidazione, ad elevate concentrazioni favorisce gli scambi molecolari promuovendo l'ossidazione, mentre a valori intermedi (prodotti ad umidità intermedia, IMF) ostacola la propagazione dei radicali liberi e lo sviluppo dell'ossidazione [1].

L'acqua negli alimenti ha un ruolo fondamentale per la sua importanza sull'organismo umano e per la capacità di rendere molte matrici alimentari gradevoli ed adatte al consumo, oltre a contribuire a mantenere nella condizione di soluti molti componenti altrimenti sgradevoli alla degustazione. Infatti, l'acqua si dispone all'interno dell'alimento aggregandosi a molti tipi di molecole, soprattutto macromolecole come carboidrati e

proteine, contribuendo alle loro caratteristiche reologiche a noi note come piacevoli, come l'elasticità (delle proteine) e la morbidezza (della parte più interna dei prodotti da forno, ecc.). Oltre a questo, è capace di sciogliere sali e sostanze organiche polari di piccola e media dimensione, favorendo la loro presenza in sistemi non adatti a contenere composti polari, stabilizzando in sospensioni, dispersioni e dispersioni colloidali.

Molto importante è l'azione sensoriale che l'acqua esercita nelle soluzioni acquose o in dispersioni nelle matrici idrofobe. La sensazione organolettica colta al gusto e legata all'odore di un alimento sono condizionate dalla presenza e dalla forma in cui si trova l'acqua nell'alimento. Il gusto viene captato dalla sequenza di contatti delle strutture molecolari presenti nell'alimento, modulata dalla loro collocazione nelle fasi acquosa ed organica, privilegiando prima il contatto con quella acquosa in relazione al fatto che le papille gustative sono bagnate di acqua (saliva).

Negli oli alimentari sono state rivelati [5] quantitativi di acqua che vanno da 300 a 2000 mg/kg, quantitativi spesso ben al di sopra dei contenuti di saturazione (valutabili in 250-300 mg/kg, in assenza di componenti minori). La spiegazione di queste quantità è data dalla presenza di una situazione di microgocce di acqua dispersa nell'olio stabilizzata dall'aggregazione-dissoluzione di un gruppo di sostanze polari, idrosolubili e/o idrocompatibili che vanno dai sali minerali, acidi liberi, digliceridi, fosfolipidi, fino a sostanze alcoliche e fenoliche (Figura 1). Si tratta di dispersione (fine emulsione o microemulsione) e non di sospensione, data l'impossibilità di separare con mezzi fisici l'acqua, insieme ai suoi componenti, dall'olio. La quantità di acqua che si trova dispersa negli oli vergini d'oliva è relativamente variabile in relazione alla tecnologia di estrazione ed alla disponibilità di componenti minori che agiscono da surfattanti. Tale dispersione si configura come struttura micellare per il comportamento che la rende stabile e le micelle hanno una dimensione talmente piccola da non essere visibili ad occhio nudo, negli oli filtrati (inferiore al micrometro e più vicina a 0,1 μm). Calore e freddo intensi operano una destabilizzazione della dispersione, anche se con meccanismi differenti, confermando che si tratta di una dispersione metastabile, cioè le micelle sono quantitativamente al di sopra del contenuto d'acqua corrispondente alla saturazione.

Al microscopio ottico molto potente (1600 ingrandimenti), nel caso di oli extravergini di oliva, si possono rilevare sia l'aspetto che le dimensioni di tali micro-micelle (Figura 1).

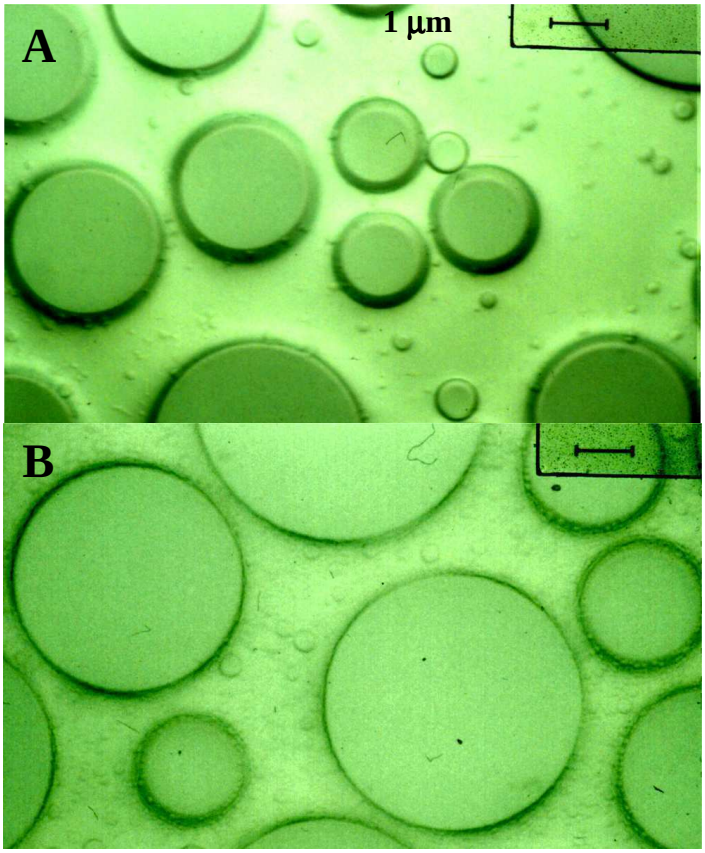

Figura 1
Microfotografia [6] di oli d'oliva durante il loro decongelamento: olio deodorato (A) e olio extravergine di oliva (B). Sono indicate alcune piccole gocce d'acqua, nei pressi del contorno di grosse gocce di olio.
Dopo solidificazione ad una temperatura inferiore a 8°C circa, l'olio viene fatto scongelare sotto un potente microscopio ottico (1600 ingrandimenti). La figura A corrisponde ad un olio deodorato "delicatamente", mentre la figura B a quello di un olio extravergine di oliva. Le frecce indicano le piccole gocce di acqua. Si possono osservare le piccolissime gocce di acqua presenti in maggiore quantità nella figura B. Le microgocce di acqua sono in prevalenza attratte dalla superficie esterna, una specie di membrana, delle gocce più grosse.

5.1.5 Indicazioni per la conservazione degli oli vergini da olive

La composizione degli oli provenienti dalla lavorazione delle olive è stata studiata da molti gruppi di ricerca nazionali ed internazionali. Tuttavia, rimangono ancora delle incognite sul comportamento dell'olio durante la conservazione, sulla complessità delle sensazioni relative all'analisi sensoriale, sulla stabilità all'ossidazione in relazione alla presenza di antiossidanti di tipo fenolico e polifenolico e sulla possibilità di rivelare la presenza di alcune particolari frodi attualmente in uso [1].

È noto da molto tempo che la stabilità di un olio che abbia subito un raffreddamento tale da solidificare, attraverso la graduale cristallizzazione dei gliceridi, quando fonderà di nuovo presenterà una minore stabilità alla conservazione.

La conservazione dell'olio è condotta a temperature basse oppure a quelle ambientali, spesso senza capire che non è casuale la serie di eventi che corrispondentemente si possono verificare. Inoltre, quando le temperatura scende al di sotto degli 8 °C l'olio vergine da olive tende a cristallizzare nei componenti trigliceridici: questo costringe tutte le altre sostanze ad attendere che i trigliceridi si esauriscano prima di solidificare, ammesso che le condizioni di temperatura lo permettano. Proprio per questa motivazione diversi componenti minori, in particolare quelli a struttura fenolica, tendono a separarsi per una buona parte, in quanto le micromicelle in cui essi sono presenti (in forma dispersa colloidale nell'olio) in virtù dell'acqua in esso contenuta, in condizione metastabile, si avvicinano in maniera determinante per la rottura di tale condizione.

I tentativi successivi di ri-solubilizzare questi costituenti precipitati in una specie di nuvola di nebbia pesante, una volta "scongelato" l'olio, sono vanificati dall'incapacità di sciogliere quelle quantità di componenti, dei quali l'olio è già saturo. L'unica possibilità sarebbe disperdere i solidi mediante omogeneizzazione, però con grave danno all'olio.

L'olio, perdendo in tal modo una parte non trascurabile di antiossidanti –nella migliore condizione di efficacia in fase dispersa- vede ridotta la sua serbevolezza originale. Oltre a ciò, l'aggregazione di componenti ricchi in acqua porterà nel tempo alla formazione della sensazioni organolettiche sgradevoli, a causa di fermentazioni anomale da parte di microrganismi presenti. Per queste motivazioni, da sempre viene consigliato separare il solido di fondo dall'olio.

Analogamente il tentativo di addizionare composti fenolici o sostanze simili in oli (vergini o di semi) non sortisce risultati interessanti in quanto i quantitativi di acqua presente o sono troppo scarsi (acqua < 500 mg/kg) o sono già saturi di sostanze disciolte o associate (acqua > 500 mg/kg). Anche l'acqua, quindi, rappresenta uno strumento per comprendere tale comportamento [1].

5.1.6. Controllo della qualità degli oli ottenuti dalle olive: caratteristiche sensoriali degli oli vergini

La maggior parte dei componenti volatili sono responsabili di molte note aromatiche, soprattutto di profumi ma anche di alcuni odori particolari (percepibili per via retro-olfattiva). Le sostanze fenoliche, invece, sono -almeno molte di esse- responsabili dei sapori degli oli vergini.

La presenza nei sistemi naturali come gli oli vergini d'oliva, ottenuti mediante tecnologie convenzionali, di acqua "tecnologica" costringe una parte di sostanze fenoliche a risiedere

in micromicelle corrispondenti all'acqua dispersa, stabilizzate proprio dalla serie di sostanze che si interfacciano a causa della loro struttura molecolare con una parte polare ed una poco polare (sciolta nella fase oleosa); ciò permette la repulsione tra le micelle in avvicinamento e la stabilizzazione della dispersione corrispondente.

Le sostanze che risiedono all'interno delle micro-micelle sono "sentite" dal punto di vista sensoriale in maniera differente da quelle sciolte nell'olio; infatti, esse sono presenti in fasi differenti e per essere percepite al momento dell'assaggio devono venire a contatto con le papille gustative dell'apparato boccale, ricoperte da un sistema acquoso (saliva). Questa situazione porta a "sentire" prima le sensazioni legate ai componenti maggiormente idrocompatibili e poi, in un secondo momento, a quelli più idrofobi (retrogusto) [1].

5.1.7. Controllo della qualità degli oli ottenuti dalle olive: ossidazione degli oli vergini d'oliva

L'ossidazione delle sostanze grasse in generale ha un andamento molto simile, ad eccezione degli oli vergini di oliva che presentano un andamento particolare. Innanzitutto, chi ha fatto studi su questo argomento ha constatato negli oli d'oliva come i tocoferoli durante la conservazione tendano a diminuire in maniera progressiva, tanto da essere considerati marcatori dell'ossidazione stessa. Con un periodo di attesa sufficientemente lungo, in condizioni di confezione sigillata, si può rilevare come essi ricompaiano in oli ossidati. In oli vecchi e già rancidi si possono infatti misurare quantitativi di tocoferoli paragonabili a quelli originalmente presenti [1].

I tocoferoli, quindi, si vanno rigenerando a spese di altri costituenti antiossidanti, che evidentemente a loro volta si ossidano. Ma perché questi ultimi non agiscono direttamente, senza scomodare i tocoferoli per questa azione, dato che poi comunque anch'essi saranno destinati ad ossidarsi?

Se si determina l'andamento dei perossidi (attraverso il numero di perossidi), ci si rende conto che il comportamento in tempi brevi assomiglia più ad un profilo a forma di sega piuttosto che ad una retta o ad una curva.

L'interpretazione di questo comportamento fino a poco tempo fa era fornita dal meccanismo cinetico che prevede un accumulo di sostanze perossidiche per avere una buona probabilità di far funzionare gli antiossidanti presenti, che abbassano la quantità di perossidi. Questo andamento ciclico potrebbe consentire di misurare un valore di perossidi maggiore un giorno e minore dopo qualche giorno, in antitesi al comportamento più logico osservato per gli oli raffinati.

La presenza delle micromicelle e quindi dell'acqua, può spiegare meglio un comportamento antiossidante vero, un po' "ritardato", considerando la necessità del verificarsi di scontri attivi con strutture (micelle) caricate parzialmente (strutture molecolari esposte dalla parte dell'olio).

Recentemente, è stato osservato che l'olio d'oliva, quando sottoposto a raffreddamento sembra ossidarsi più velocemente a bassa temperatura (se l'olio è parzialmente cristallizzato) che a temperature maggiori: esattamente all'opposto di quanto ci si attenderebbe. L'interpretazione fornita si basa sul fatto che a bassa temperatura gli antiossidanti si separano dall'olio e non possono più svolgere la loro azione antiossidante, aggravata dal fatto che a bassa temperatura cristallizzano prima gli acidi grassi meno insaturi, residuando una composizione pertanto più ossidabile. Pur ritenendo che non si possano escludere questi due aspetti, si sa che la cristallizzazione avviene in "purezza", cioè fra gliceridi più simili a partire da quelli più rappresentati: questo comportamento prevede un effetto di concentrazione di tutte le sostanze definite componenti minori. Fra questi, le micromicelle rappresentano una situazione che va deteriorandosi mano a mano che queste sono costrette ad avvicinarsi fino a collassare. Tale situazione si risolverà con una perdita di fenoli e polifenoli, come già detto. La considerazione è valida anche per i perossidi –gli idroperossidi degli acidi grassi insaturi, per l'esattezza- che tenderanno ad incrementare esasperando le condizioni ossidative.

Quindi, più che diminuzione dei fenoli antiossidanti ed insaturazione aumentata, sarebbe l'aumento di concentrazione dei perossidi in un compartimento ridotto la causa più probabile del comportamento sperimentale osservato. Oltre a ciò si potrebbe considerare l'effetto chimico fisico di "espulsione" dell'esanale, analiticamente misurata come indice di ossidazione, dovuta alla progressiva riduzione degli spazi e all'eccessiva sua concentrazione (sovrasaturazione) [1].

5.1.8. Controllo della qualità degli oli ottenuti dalle olive: recenti frodi nel settore degli oli provenienti dalla lavorazione delle olive

L'ultima frode che è stata considerata problematica da studiare ha riguardato la riduzione o eliminazione di sostanze maleodoranti da oli che, in seguito al trattamento migliorano le proprie condizioni commerciali (passando a categorie merceologiche di livello superiore). Questo procedimento, fraudolento se applicato a oli commercializzati come vergini o che saranno miscelati con altri per essere venduti come vergini, quando è condotto in condizioni blande ("deodorazione soft o delicata") non lascia segni di facile individuazione.

La deodorazione, da quel po' che si può sapere a riguardo, sembra venga condotta attraverso sistemi differenti: con una specie di distillazione molecolare, condotta sotto vuoto e a bassa temperature, o mediante gorgogliamento con azoto o vapor d'acqua a circa 100 °C, oppure con l'impiego di polveri adsorbenti o ancora con l'impiego di membrane filtranti.

Dopo diversi anni di ricerche [5] di marker in grado di individuare tale frode, finalmente nel 2006 il gruppo di ricercatori dell'Instituto de la Grasa di Sevilla coordinati dal dott. Arturo Cert Presentano al Congresso della Federazione Europea di Scienza e Tecnologia dei Lipidi (EUROFED LIPID) l'analisi degli alchil esteri [7].

5.1.9. Genuinità e qualità

Spesso la qualità di un olio ottenuto dalle olive è confusa con la genuinità. Un olio che corrisponda a quello indicato in etichetta è genuino, ma non è automaticamente di qualità.

Nel caso degli oli da olive la genuinità è molto importante, perché per tutte le altre sostanze grasse i prezzi sono abbastanza vicini da non rendere economicamente interessante la frode.

La genuinità degli oli da olive è controllabile impiegando le determinazioni analitiche previste per i parametri corrispondenti dal Reg. CEE 2568 del 1991 e successive modificazioni [4].

5.1.10 Composti gliceridici

I maggiori costituenti di una sostanza grassa alimentare sono composti di natura gliceridica: in particolare, normalmente, dominano i trigiceridi (triacilgliceroli) fino oltre al 95% circa, poi vi sono digliceridi (diacilgliceroli) fino al 2-4% ed infine i monogliceridi (monoacilgliceroli). L'analisi dei costituenti gliceridici è importante per vari aspetti: alcuni di essi sono caratterizzanti per la valutazione della qualità delle materie prime (digliceridi totali), alcuni lo sono per la rivelazione di frodi (2-monogliceridi), alcuni altri per la valutazione della freschezza (1,2-digliceridi/1,3-digliceridi)) e altri ancora per la previsione della conservazione (trigliceridi ossidati). Dal punto di vista diagnostico per il controllo della genuinità sono importanti gli acidi grassi, tanto da essere determinati come composizione da tutti coloro che esaminano le sostanze grasse alimentari [1].

Analisi dei diacilgliceroli (digliceridi): La quantità totale dei diacilgliceroli (DG) è in relazione al livello di idrolisi della sostanza grassa, sia subito a livello di materia prima (conservazione) sia incrementata dalla sua trasformazione. Attraverso l'idrolisi si forma anche acidità libera, normalmente misurata come parametro di qualità, ma nel caso di oli

che vengano poi raffinati, rimangono come testimoni dell'inacidimento solo i digliceridi. Oggi sono possibili analisi che realizzano ottime separazioni basate sulla dimensione e sul grado di insaturazione totale del singolo digliceride, si è potuto mettere a punto un metodo di determinazione dei digliceridi per la misurazione anche degli 1,2-DG separatamente dagli 1,3-DG. Con tale possibilità analitica si è evidenziata l'importanza della determinazione del rapporto fra i due tipi di digliceridi nella valutazione della qualità degli oli di oliva. Tale rapporto diminuisce sempre di più in quanto gli 1,3-DG incrementano, nel tempo di conservazione, per effetto idrolitico selettivo dei TG e per isomerizzazione dagli 1,2-DG [1].

Analisi dei monoacilgliceroli (monogliceridi). La determinazione della composizione degli acidi grassi presenti nella posizione centrale della glicerina, dopo degradazione con lipasi pancreatica dei trigliceridi di origine vegetale, è utile all'individuazione di sostanze grasse esterificate chimicamente. L'analisi diretta dei monogliceridi (come trimetilsilil derivati, TMS) nel lipolizzato ha permesso un notevole risparmio di tempo e una migliore misura a causa della diminuzione delle interferenze, spesso riscontrate per il metodo ufficiale di analisi precedenti [1].

5.1.11 Analisi degli acidi grassi

La determinazione degli acidi grassi è la più classica delle determinazioni gas cromatografiche: oggi si preferisce affrontare oltre alla determinazione degli acidi grassi totali o di quelli relativi a particolari frazioni lipidiche, in diversi casi, la determinazione degli acidi grassi liberi, previo trattamento metilante con diazometano. Rimane importante ricordare, tuttavia, che quando la composizione di un olio, o di lipidi in generale, non è stata ampiamente studiata, è bene approfondire in via preliminare la distribuzione dei costituenti anche mediante una cromatografia su strato sottile (TLC) di tipo analitica, dalla quale può emergere la presenza di derivati degli acidi grassi, ossidati o più polari.

Nel caso della determinazione della composizione degli acidi grassi (sotto forma di esteri metilici) è stato ampiamente sfruttato l'impiego di colonne capillari polari, che forniscono la possibilità di separare abbastanza bene anche gli acidi grassi *trans*-isomeri, al punto da diventare un metodo della CE per il controllo della genuinità degli oli vergini (che non hanno subito raffinazione) [1].

5.1.12 Determinazione della composizione degli acidi grassi

La composizione degli acidi grassi consente di svelare le frodi più grossolane, in quanto è la determinazione che viene condotta più di frequente e, quindi chi froda, la conosce e ne rispetta le possibilità diagnostiche, operando frodi che non siano facilmente svelate da questa analisi. Tuttavia, nella determinazione della composizione degli acidi grassi è possibile valutare anche gli acidi grassi in configurazione *trans*, con particolare interesse per quelli monoinsaturi (monoeni). Tali isomeri *trans* sono la traccia di raffinazione degli oli e, quindi la loro presenza è indice -come le determinazioni spettrofotometriche- di miscele con oli raffinati [1].

5.1.13 Determinazione dei componenti dell'insaponificabile (liberi e combinati)

I componenti "minori" degli oli, soprattutto quelli dell'insaponificabile sono da sempre stati considerati molto informativi sulle miscele fraudolente degli oli d'oliva. I metodi più interessanti sono quelli basati sui componenti dell'insaponificabile liberi ed esterificati, determinati mediante analisi separate, con particolare riferimento per gli steroli [8]. Questo metodo consente di rivelare piccole presenze di olio di nocciola, anche se sottoposto preventivamente a deodorazione spinta per eliminare i componenti liberi dell'insaponificabile (che di solito sono maggioritari).

Oltre a ciò, la validità delle determinazioni sui componenti dell'insaponificabile sta nella capacità di indicare la presenza di determinati oli nelle miscele con oli d'oliva, anche in relativamente piccole quantità.

La determinazione quali-quantitativa degli stereni, con particolare interesse per gli steradieni, fa parte delle determinazioni utili a svelare la miscela di oli raffinati negli oli vergini, quella più sensibile.

Recentemente sono state proposte delle condizioni d'analisi per i componenti dell'insaponificabile che richiamano la logica dei "finger print". Si tratta di una specie di "insapogramma" (cromatogramma della frazione insaponificabile), i cui costituenti dal punto di vista quali-quantitativo possono indicare una situazione anomala molto rapidamente e, in questi casi, imporre altri tipi d'indagine. Nella maggior parte dei casi non sarà necessario procedere con ulteriori analisi, risparmiando onerosi impegni d'analisi [1].

5.1.14 Resistenza all'ossidazione forzata

Fra i vari sistemi di valutazione della situazione ossidativa di una sostanza grassa, i test di resistenza ad ossidazione accelerata o di resistenza al maltrattamento termo-ossidativo,

sembrano quelli più efficaci, anche se sono criticabili per numerosi aspetti. Lo scopo è quello di ottenere una misura obiettiva della sua qualità in relazione alla stabilità durante la conservazione. Sono disponibili alcune apparecchiature automatizzate capaci di fornire un dato di stabilità molto utilizzato per caratterizzare una sostanza grassa e, in alcuni casi, diagnostico per capire l'influenza di alcuni parametri di produzione sulla qualità di un olio.
Questa determinazione si riferisce ad un aspetto della qualità intrinseca che però non ha avuto ancora impieghi ufficiali [1].

5.1.15 Composti fenolici

La determinazione dei componenti con struttura molecolare fenolica e più nello specifico *o*-difenolica per via della loro maggiore attività [9] ha lo scopo di qualificare la capacità antiossidante dell'olio e quindi la sua futura stabilità durante la conservazione [10]. Pertanto, la determinazione chimico-analitica dei composti fenolici e della loro attività antiossidante ha caratteristiche simili a quella più empirica della resistenza all'ossidazione forzata, una determinazione utile a stabilire la qualità intrinseca dell'olio.

5.1.16 Prodotti d'ossidazione dei fenoli e polifenoli

Questa valutazione analitica non ha ancora avuto una definizione nella forma di metodo analitico, tuttavia, la presenza di prodotti di ossidazione di tali molecole è inevitabile in un olio. Essi derivano dal contatto olio pasta, ma anche dalla protezione che i polifenoli esercitano nel tempo di conservazione. Determinare i fenoli ossidati, insieme alla misura totale dei fenoli e polifenoli (o degli *o*-difenoli) significa poter mettere in relazione questi dati con la reale capacità antiossidante. Infatti, il potere antiossidante complessivo è condizionato dagli equilibri che si instaurano fra i componenti redox, ovvero tra fenoli presenti in forma ridotta (attiva) ed in forma ossidata.
In ogni caso, quando si potrà disporre di un metodo analitico per i prodotti d'ossidazione dei fenoli, la determinazione sarà rivolta all'aspetto della qualità intrinseca del prodotto, o meglio alla sua stabilità nel tempo [1].

5.1.17 Componenti volatili

Le sostanze volatili costituiscono in generale i componenti odorosi di un alimento e, nel caso degli oli vergini d'oliva, sono di particolare importanza per il giudizio sensoriale. La disponibilità di una metodica di analisi dei volatili degli oli vergini, oltre a consentire un controllo quali-quantitativo dei componenti responsabili di pregi e difetti percepibili

all'olfatto, è anche utile in un controllo anti-frode, in quanto permette il contemporaneo dosaggio del contenuto di composti benzenici e di idrocarburi alogenati [1].

5.1.18 Le verifiche per genuinità del Parmigiano-Reggiano e per altre tipologie di formaggi

Come noto ai consumatori, e come ben illustrato da Bertozzi e Bocedi in un recente volume del mensile L'Agricoltura [11], la fama acquisita dal Parmigiano-Reggiano durante i suoi nove secoli di vita ha portato a molteplici tentativi di imitazione con lo scopo di sfruttare in modo illecito il suo valore. Pertanto, nel corso dei secoli sono state messe in atto diverse iniziative per garantire l'origine del prodotto, così come tale prodotto ha rappresentato un esempio oltre che il precursore degli attuali sistemi di tutela dei prodotti legati ad una specifica zona di origine. Infatti, il duca di Parma Filippo di Borbone con una "grida" del 16 giugno 1751 imponeva sin da allora di bollare i formaggi prodotti nel Parmense. Si arriva quindi ai primi anni del 1900 quando si realizzarono azioni congiunte che portarono prima alla tutela del Grana Tipico, poi del Parmigiano-Reggiano con un ambito di protezione molto ampio. Nello specifico, già nel 1909 i rappresentanti delle Camere di commercio di Parma, Reggio Emilia, Modena e Mantova avviarono un'azione per un'unica organizzazione di "marcatura del grana" che portò nel 1921 alla costituzione di un comitato interregionale con lo scopo di identificare il "formaggio Reggiano tipico", apponendo su ogni forma una marca d'origine a garanzia dei caratteri tipici esterni ed interni del formaggio "Grana Reggiano". Successivamente, nel 1934 venne costituito il Consorzio Volontario Interprovinciale del Grana Tipico, che comprendeva la "zona veramente classica della produzione dello squisito e rinomato formaggio universalmente noto sotto il nome di reggiano e di parmigiano" che nel 1937 veniva estesa alla provincia di Bologna in sinistra Reno, con lo scopo di difendere, tutelare e promuovere il prodotto [11].

Dopo la seconda guerra mondiale, nel 1951 venne sottoscritta a Stresa fra i rappresentanti di Italia, Francia, Svizzera, Austria, Danimarca, Norvegia, Paesi Bassi e Svezia la prima Convenzione Internazionale sull'impiego delle *denominazioni d'origine* e *denominazioni dei formaggi*, che portò poi alla legge italiana n. 125 del 1954 sulla tutela dei formaggi, suddivisi in denominazioni d'origine e tipiche [11].

La denominazione Parmigiano-Reggiano venne riconosciuta l'anno successivo e il Consorzio del Parmigiano-Reggiano svolse un ruolo di primo piano per ottenere tali provvedimenti, in virtù della necessità di riconoscere la denominazione a livello nazionale ed internazionale. La normativa nazionale istituì anche specifiche disposizioni e sanzioni

penali contro le frodi e la concorrenza sleale per la tutela delle denominazioni d'origine dei formaggi e, infine, sostituite dal Regolamento (CEE) 2081/92, oggi Regolamento (CE) 510/06 [12]. Tale normativa conferisce un'ampia difesa alle denominazioni registrate, in quanto specificava che le stesse erano tutelate contro qualsiasi usurpazione, imitazione ed evocazione, anche se tradotte e anche se la vera origine del prodotto era indicata, protezione mantenuta dell'attuale Reg.(CE) 510/06 e che va al di là del rischio di confusione del consumatore, come poi ribadito in reiterate occasioni dalla Corte di Giustizia Europea.
A tutela del prodotto processato e confezionato, il DPR 22 settembre 1981 stabilì che i formaggi a denominazione d'origine potevano essere commercializzati anche in parti preconfezionate e che sugli involucri delle stesse doveva essere estesa l'apposizione della denominazione d'origine. Infine il DPCM 4 novembre 1991 estese l'uso della denominazione di origine del formaggio «Parmigiano-Reggiano» anche alla tipologia «grattugiato».
Alla stregua del Parmigiano-Reggiano, anche altri formaggi che vantano il riconoscimento della Denominazione di Origine Protetta, possono beneficiare delle tutele legate a questo marchio Europeo. Tra i Pecorini vantano tale riconoscimento il Pecorino Sardo, il Pecorino Romano, il Pecorino Toscano, il Pecorino Siciliano, il Fiore Sardo, il Canestrato Pugliese ed il formaggio di Fossa di Sogliano al Rubicone e Talamello). Tali formaggi sono caratterizzati dall'utilizzo di una materia prima che determina specifiche caratteristiche sensoriali e compositivo oltre all'effetto del processo di produzione che caratterizza ciascuno di questi.

5.2 Metodi di analisi fisici: la spettrometria nell'infrarosso e calorimetria a scansione differenziale

5.2.1 Principi teorici di spettroscopia IR

La spettroscopia è la scienza che studia l'interazione tra la radiazione elettromagnetica e la materia. In particolare, la spettroscopia, è utilizzata per indicare la separazione, rivelazione e registrazione di variazioni di energia (picchi di risonanza) che interessano nuclei, atomi o intere molecole. Queste variazioni energetiche sono dovute all'interazione tra radiazione e materia, specificatamente all'emissione, assorbimento o diffusione di radiazioni o particelle elettromagnetiche [13].

La base teorica di tale interazione è la natura quantica del trasferimento di energia dal campo di radiazioni alla materia e viceversa. Infatti, sia la materia che il campo elettromagnetico hanno una "doppia natura", ossia la possibilità di comportarsi sia come onda che come particella.

La radiazione elettromagnetica è una forma di energia che si propaga attraverso lo spazio ad altissima velocità come un'onda e si può presentare sotto numerose forme, fra cui una delle più comuni è la luce.

Essa è la risultante della sovrapposizione di un campo elettrico e di un campo magnetico ortogonali mutuamente accoppiati: ciascuno dei due è sorgente dell'altro e si propaga con andamento sinusoidale sia nello spazio che nel tempo. (figura 2)

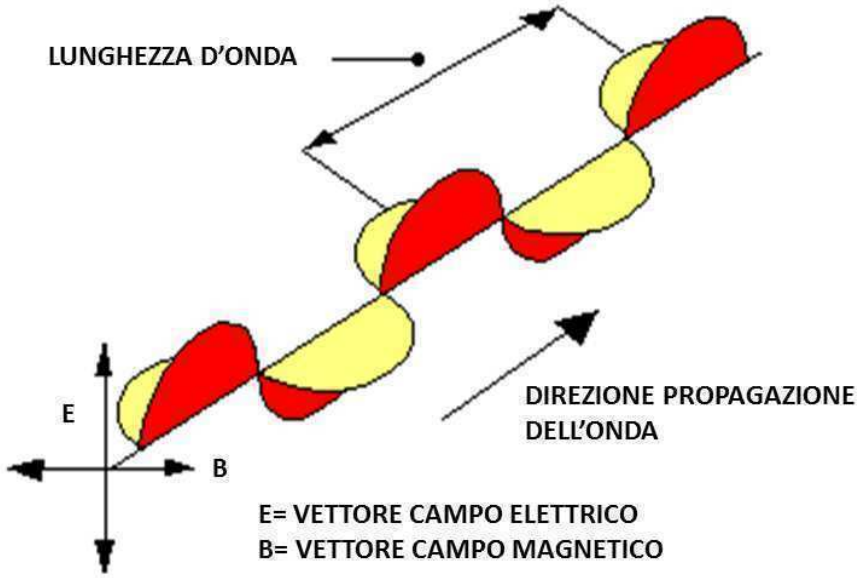

Figura 2
Rappresentazione di un'onda elettromagnetica

Il carattere ondulatorio della radiazione elettromagnetica è comunemente descritto da:

- **lunghezza d'onda (λ)**, che rappresenta la distanza tra due massimi adiacenti e viene misurata in nm;
- **frequenza (*v*),** che rappresenta il numero di oscillazioni descritte dall'onda nell'unità di tempo ed è misurata in Hertz (cicli/s);
- **numero d'onda (σ)**, che rappresenta il numero di onde presenti in un'unità di lunghezza ed è misurato in cm^{-1};
- **velocità di propagazione (c)**, che è la velocità con la quale avanza il fronte d'onda e che nel vuoto, per tutte le frequenze, assume il valore di $2{,}998 \cdot 10^{10}$ cm/s.

Lo spettro della radiazione elettromagnetica è composto da diverse zone delimitate da lunghezze d'onda precise. Questa suddivisione origina cinque grandi gruppi: la frazione visibile, che occupa la parte centrale dello spettro ed ha caratteristiche intermedie, la frazione ultravioletta e i raggi ionizzanti, caratterizzati da alte frequenze e corte lunghezze d'onda, ed infine la frazione infrarossa e le onde radio, caratterizzate da bassa frequenza ed alte lunghezze d'onda (figura 3).

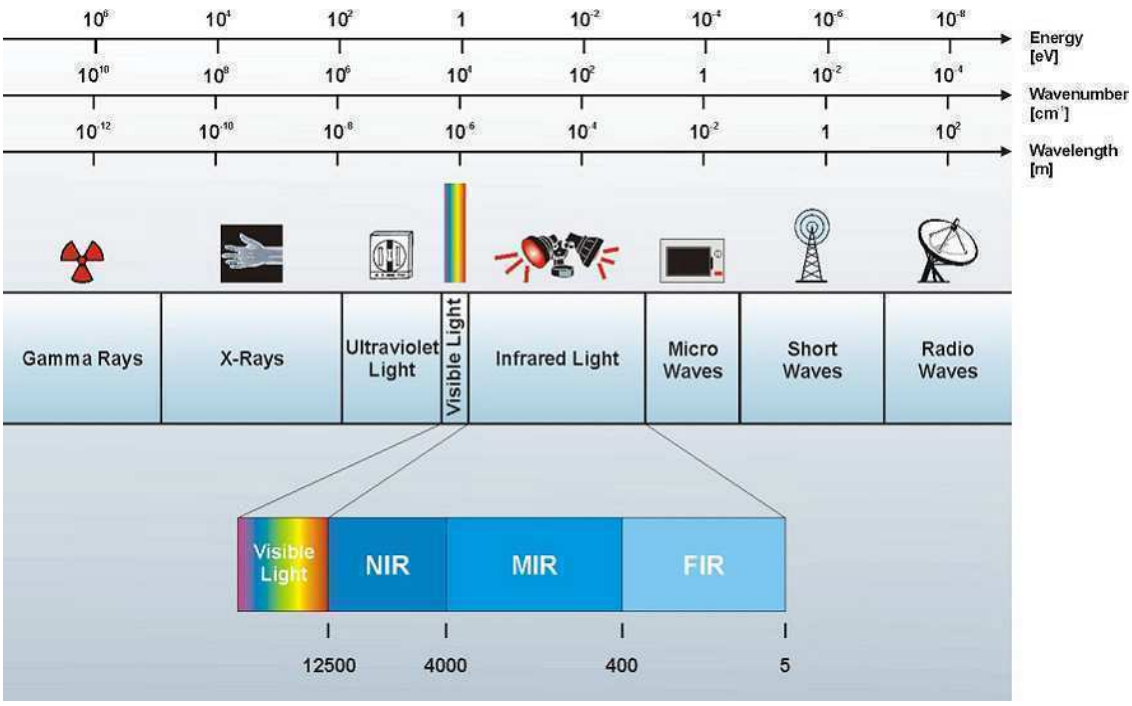

Figura 3
Rappresentazione grafica delle zone dello spettro elettromagnetico

La radiazione rivela la sua natura corpuscolare quando interagisce con la materia. Essa non trasmette una quantità di energia continua ma trasmette "pacchetti" di energia quantizzati; può essere quindi vista come un flusso di particelle dette fotoni.

Quando l'energia di una radiazione va incontro a quella di una molecola che sta vibrando, c'è un trasferimento netto di energia che può avvenire solo ed unicamente se il fotone ha una frequenza, e quindi un'energia, uguale a quella necessaria per far passare la molecola dallo stato fondamentale a quello eccitato.

Ciò può essere rappresentato come variazione di energia in ordinata e lunghezza d'onda in ascissa, ovvero come uno spettro (figura 4).

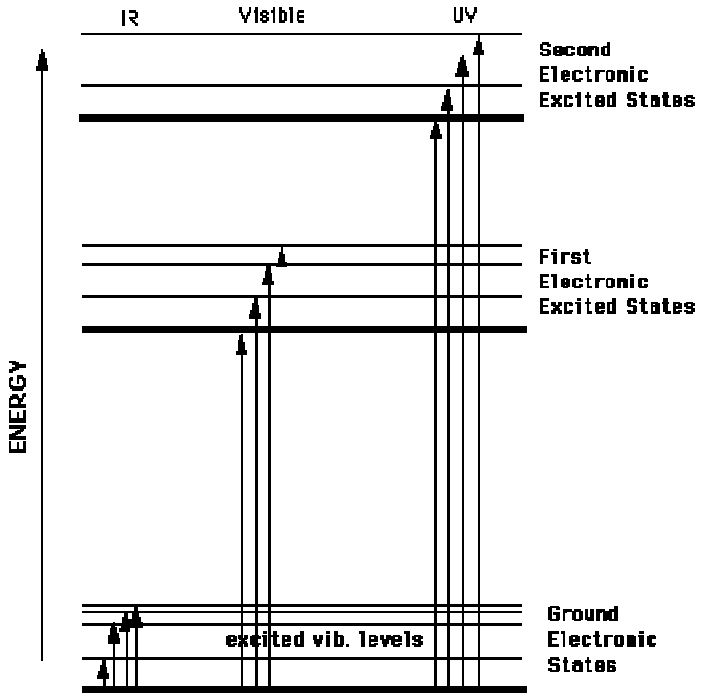

Figura 4
stati energetici quantizzati di un atomo

L'energia più bassa corrisponde alla configurazione più stabile detta "configurazione di base", il livello successivo corrisponde al primo livello eccitato.

Se un fotone di energia uguale al dislivello tra le due configurazioni considerate colpisce una molecola, un elettrone che si trova allo stato di base possiede una certa probabilità di portarsi allo stato eccitato: il fotone è stato assorbito. Dopo un certo tempo, tipicamente di 10^{-8} secondi, l'elettrone ritorna allo stato base ed emette un'energia uguale al salto di energia tra i due livelli.

Conoscendo l'intensità dalla radiazione incidente e di quella emessa, si può risalire alla frequenza della radiazione che è stata assorbita e quindi al salto energetico a cui è andata incontro la molecola. Infine, sapendo che i salti con un dato livello energetico possono interessare solo certe molecole, si può capire quali molecole costituiscono la materia. Per questi motivi, l'assorbimento o l'emissione di energia da parte della materia è uno dei più importanti marchi d'identificazione forniti dalla natura [13].

Una radiazione della frazione IR dello spettro è caratterizzata da una bassa energia; i fotoni non sono in grado di eccitare la molecola ma possono indurre moti vibrazionali degli elettroni in modo tale che il suo momento dipolare elettrico cambia durante la vibrazione.

I fondamentali tipi di vibrazione causati dall'incidenza di una radiazione della frazione **IR** dello spettro sono:

- ***stretching*** (stiramento), ovvero la vibrazione del legame lungo il piano, in conseguenza al quale varia ritmicamente la distanza interatomica e può essere simmetrico o asimmetrico;
- ***bending*** *(*deformazione) ovvero un movimento a forbice, caratterizzato da una variazione dell'angolo tra due atomi nel piano (scissoring e rocking) e fuori piano (wagging e twisting).

5.2.2 Spettroscopia nel vicino infrarosso (NIR)

La spettroscopia del vicino infrarosso analizza gli stati vibrazionali delle molecole utilizzando onde elettromagnetiche con un'energia dei fotoni nell'intervallo tra 2,65 10^{-19} e 7,96 10^{-20} J a cui corrisponde una lunghezza d'onda tra 780 e 2500 nm.

Nella zona dello spettro elettromagnetico definita come vicino infrarosso (NIR), le energie in gioco sono atte a determinare una variazione nel moto vibrazionale delle molecole e in particolare dei legami in esse presenti. Gli assorbimenti degli stati fondamentali cadono quasi sempre nella regione tra 2500 e 15000 nm (4000-660 cm^{-1}) definita come medio infrarosso (MIR), mentre quelli degli stati con frequenze multiple a quella dello stato fondamentale, detti overtones, sono caratteristici della zona del NIR.

La spettroscopia nel vicino infrarosso permette, quindi, di ottenere un'analisi di tipo quantitativo nel determinare componenti che contengono legami **C-H**, **N-H**, **O-H** ad esempio per la misura di acqua, fenoli, alcoli, ammine ed idrocarburi, consentendo sia di effettuare un'analisi diretta sul campione sia di ottenere uno spettro caratteristico del campione.

Le misure NIR possono essere eseguite sia in trasmittanza che in riflettanza; nel caso dell'acquisizione dei dati in trasmittanza ciò che viene misurato è l'intensità della luce trasmessa attraverso il campione rispetto all'intensità della luce incidente:

$$T = I/I_0$$

Lambert e Beer osservarono che la quantità di radiazione assorbita o trasmessa da una soluzione o da un mezzo era funzione esponenziale della concentrazione della sostanza assorbente contenuta e della lunghezza del cammino percorso dalla radiazione attraverso il campione.

Integrata per uno spessore finito, secondo la legge di Lambert e Beer, è definita assorbanza di un campione:

$$A = log1/T = k_a cl$$

dove K_a è il coefficiente di estinzione molare, c è la concentrazione ed l è la lunghezza del cammino ottico della radiazione incidente attraverso il campione.
Nel caso di acquisizione di dati in *riflettanza*, ciò che viene misurato è l'intensità della luce riflessa rispetto all'intensità della luce incidente:

$$R = I_{rifl} / I_0$$

Secondo la legge di Kubelka-Munk, la riflettanza dipende dal coefficiente di assorbimento K_R e dal coefficiente di dispersione s di un campione:

$$f(R_\infty) = K_R / s$$

dove R_∞ è la riflettanza assoluta.

A livello sperimentale viene normalmente misurata la riflettanza relativa, cioè l'intensità della luce riflessa dal campione rispetto all'intensità della luce riflessa da un materiale di riferimento, con una riflettanza assoluta alta e costante (teflon, MgO, dischi di materiale ceramico). Nella pratica, la riflettanza relativa è spesso convertita in assorbanza apparente A', utilizzando una relazione empirica tra concentrazione di analita e riflettanza simile alla legge di Lambert e Beer:

$$A' = log\ 1/R = a' c$$

Tuttavia, se la matrice è altamente assorbente o l'analita mostra bande di assorbimento piuttosto intense la relazione lineare tra assorbanza e concentrazione viene meno. Sia per la trasmittanza che per la riflettanza le equazioni proposte sono ricavate da situazioni ideali e sono applicabili solamente quando gli assorbimenti sono deboli o il prodotto tra concentrazione e coefficiente di estinzione molare è piccolo. Nel caso della spettroscopia NIR, l'assorbimento avviene da parte della matrice, che non è separabile dall'analita, e può assorbire alle medesime lunghezze d'onda del composto in esame.
L'interazione con la materia può avvenire quindi in diversi modi, ovvero può essere assorbita dal campione, riflessa, oppure trasmessa in parte o completamente attraverso il campione. Il modo ed il grado con cui questi effetti avvengono dipendono dallo stato fisico del campione e dal sistema di lettura utilizzato. Capire queste interazioni è importante per

comprendere le potenzialità di uno strumento NIR. I sistemi a trasmissione sono usati principalmente per campioni allo stato liquido o per strati sottili di solidi, mentre la modalità di riflessione della radiazione è più utile per campioni allo stato solido. Se il campione non riflette e non trasmette la radiazione in modo sufficiente, è possibile utilizzare come parametro la misura della trasflettanza. In questo caso la radiazione penetra nel campione, che ne assorbe una parte, poi viene riflessa su una superficie non assorbente posta sul fondo della cella e ritrasmessa attraverso il campione al detector.

Sono attualmente disponibili diverse tipologie di sistemi di presentazione, strettamente dipendenti dalla tecnologia di costruzione dello strumento.

Spesso poi la spettroscopia è affetta da fenomeni di scattering, ovvero di diffrazione della radiazione sulla superficie, specialmente nel caso dell'acquisizione su campioni solidi: infatti, tanto più si ha scattering della radiazione incidente, tanto meno in profondità penetra il raggio e perciò tanto minore sarà l'assorbanza (apparente o reale). I fenomeni di scattering dipendono essenzialmente dalle proprietà fisiche del campione (dimensione delle particelle, ambiente cristallino) e possono provocare spostamenti nella linea di base dello spettro e indurre fenomeni di col linearità alle diverse lunghezze d'onda [14].

La dipendenza del segnale dalle proprietà fisiche del campione, che è molto utile quando la tecnica NIR è impiegata per determinare parametri fisici, è un notevole svantaggio quando è utilizzata per determinazioni qualitative come l'identificazione di prodotti o il monitoraggio di parametri chimici di un processo (umidità, omogeneità dei campioni), e per l'analisi quantitativa di uno o più componenti. Per ovviare a ciò, sono stati messi a punto alcuni pretrattamenti di tipo matematico da eseguirsi sugli spettri prima dell'elaborazione statistica vera e propria.

5.2.3 Strumenti

Un generico spettrometro che emette nella regione dell'infrarosso, è costituito da un gruppo d'elementi di base: una sorgente di radiazioni, un selezionatore di lunghezze d'onda, un sistema di esposizione del campione alla radiazione e un detector, secondo la schema illustrato in figura 5:

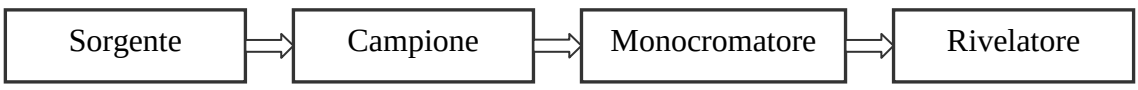

Figura 5
Schema di uno spettrometro

5.2.4 Tecniche di campionamento

La strumentazione NIR è in continua crescita, grazie allo sviluppo di fibre ottiche che permettono di acquisire gli spettri in modo diretto e semplice ponendo la punta della fibra stessa sulla superficie e/o all'interno del campione.

In caso di misure su solidi disomogenei, invece, il sistema di campionamento più adatto è quella della sfera integratrice, il cui principio di funzionamento è illustrato in figura 6.

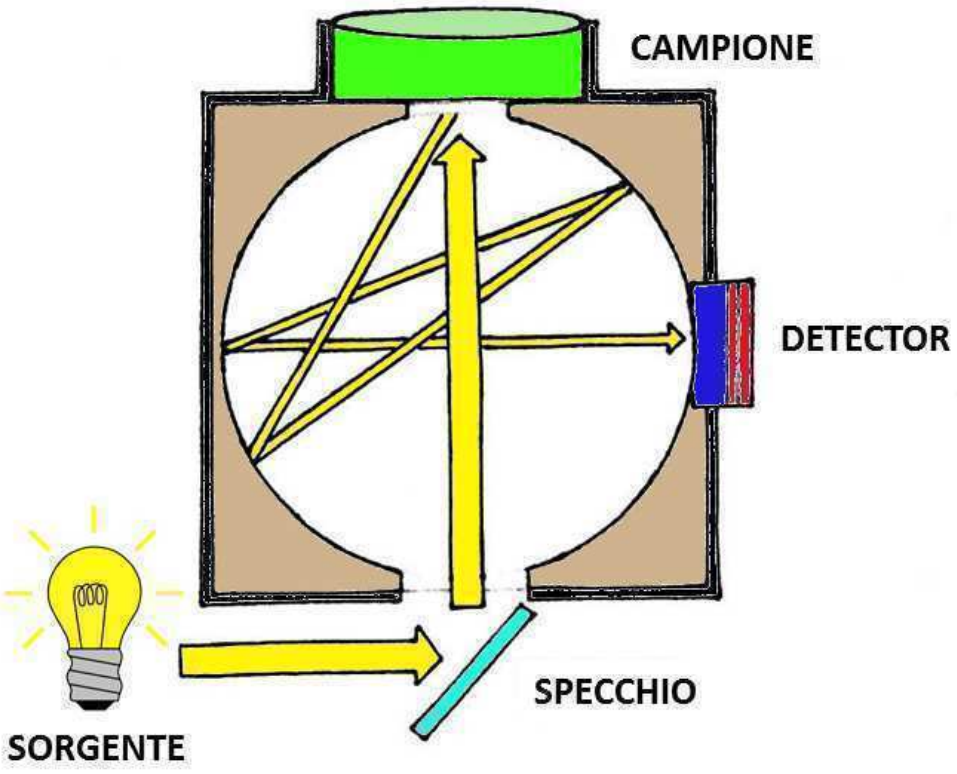

Figura 6
Principio di funzionamento di una sfera integratrice.

La radiazione colpisce uno specchio esterno alla sfera che a sua volta la indirizza sul campione. La parte di radiazione non assorbita dal campione è riflessa verso la superficie interna della sfera ed è raccolta dal detector. La sfera è ricoperta interamente in oro, ha un'area di misura di 15 mm di diametro e ha un sistema di riferimento interno. Il campione viene generalmente raccolto in un contenitore con fondo trasparente alla radiazione e dotato di una geometria tale da permettere anche la rotazione del campione, operazione necessaria quando si lavora con campioni non omogenei.

La spettroscopia NIR è largamente impiegata nell'industria alimentare per l'analisi compositiva centesimale di materie prime, intermedi di lavorazione, così come di prodotti finiti.

5.2.5 La spettroscopia nel medio infrarosso (MIR)

La spettroscopia nel medio infrarosso (MIR, anche se spesso indicato come IR) è una tecnica basata sull'assorbimento di radiazioni elettromagnetiche nella regione dello spettro di numeri d'onda compresi tra 4000 e 400 cm^{-1}.

Lo spettro di un prodotto alimentare acquisito mediante spettroscopia MIR è in grado di rivelare informazioni sulla natura dei legami presenti all'interno del campione e quindi sulla sua struttura molecolare. Tale tecnica spettroscopica può pertanto trovare impiego sia nell'analisi quantitativa che nell'analisi qualitativa di un prodotto alimentare. La spettroscopia nel medio infrarosso ha avuto un largo impiego nel settore dell'industria farmaceutica al fine dell'identificazione di composti chimici, grazie allo stretto legame esistente tra la natura chimica della materia e la sua capacità di assorbimento delle radiazioni MIR. L'esposizione dei composti ai raggi infrarossi provoca infatti l'eccitazione dei legami covalenti presenti all'interno della molecola e il conseguente salto energetico dallo stato fondamentale della struttura molecolare a quello eccitato.

La lunghezza d'onda alla quale si verifica una vibrazione molecolare può essere facilmente associata ad una determinata tipologia di legame, nonché ad una specifica modalità vibrazionali (di streching o bending). L'energia vibrazionale è inoltre strettamente correlata alla forza di legame e alla massa del sistema molecolare. Sono queste le caratteristiche che permettono l'identificazione di specifiche identità chimiche. In tabella 1 sono riportate le frequenze di assorbimento della radiazione MIR di alcuni gruppi molecolari.

Tabella 1: Frequenze di assorbimento della radiazione MIR di alcuni gruppi molecolari

Numero d'onda (cm^{-1})	Gruppo funzionale	Modalità vibrazionale	Costituente
Regione delle impronte digitali			
1036,1088	C-O	Stretch	
1060	C-O	Stretch	Carboidrati
900-1200	C-O, C-C, O-H	Stretch	Carboidrati
1115-1170	C-O	Stretch	
1232	C-H	Bend	
1240	C-O	Stretch	
1371	C-H	Bend	
1274,1372, 1445, 1486	O-C-H, C-C-H, C-O-H	Bend	
1400, 1477	C-H	Bend	
Regione dei gruppi funzionali			
1535-1570	Amido II	Stretch	Proteine
1620-1690	Amido I	Stretch	Proteine
1640	O-H	Bend	Umidità
1600-1900	O-H	Bend	Ac. Organici
1700-1765	C=O	Stretch	Lipidi
2869	CH_2	Stretch	Lipidi
2926	CH_3	Stretch	Lipidi
3047-3703	O-H	Stretch	Umidità

I recenti progressi nell'ambito della spettroscopia MIR (spettroscopia FT-IR) e le relative tecniche di "presentazione" dei campioni (cristalli in riflettanza attenuata) hanno condotto al crescente interesse verso tale tecnica in quanto si tratta di una metodica rapida, non distruttiva e relativamente economica che produce un grandissimo numero di informazioni attraverso un'unica analisi.

5.2.6 Lo strumento FT-IR

Il principale componente di uno spettrometro FT-IR è l'interferometro. Il segnale che arriva al detector produce un interferogramma che contiene le informazioni spettrali riguardanti il campione. L'interferogramma è quindi facilmente digitalizzato attraverso l'applicazione di un algoritmo matematico (la trasformata di Fourier): in questo modo si ottiene un grafico delle assorbanze tipiche di ogni legame molecolare presente nella sostanza analizzata per ottenere un incremento del segnale rispetto al rumore di fondo si opera generalmente una media tra più di un interferogramma.

Prima di analizzare ogni campione, si raccoglie uno spettro di background attraverso un'analisi in bianco. Rapportando ogni singola analisi al relativo background si produce uno spettro di assorbanza o trasmittanza, nel quale il rumore di fondo legato alle condizioni esterne è eliminato.

Un altro vantaggio correlato all'utilizzo della spettroscopia FT-IR è che tale sistema è calibrato al fine di provvedere ad una stabilità duratura dello strumento.

Le tecniche di presentazione dei campioni per la valutazione in trasmissione sono sempre state le più comunemente impiegate per il campionamento e l'analisi di gas, liquidi, paste e polveri. Tuttavia, grazie alla facilità di collocazione del campione e di pulizia dello strumento, la tecnica della riflettanza totale attenuata (ATR) è divenuta il metodo di più largo impiego per l'analisi delle sostanze liquide, delle paste e persino delle polveri.

Tale metodica si basa sul principio di trasmissione della radiazione attraverso un elemento ottico chiamato IRE (elemento interno di riflettanza) [15].

In questo sistema, il raggio luminoso attraversa il cristallo ATR e viene riflesso all'interfaccia cristallo/campione; l'angolo di incidenza impiegato permette di ottenere più di una riflessione del raggio ad opera del campione.

In ogni punto in cui le radiazioni colpiscono l'interfaccia cristallo/campione, esse penetrano per una distanza di circa 2 µm all'interno della sostanza in analisi e subiscono un decadimento logaritmico "come un'onda" all'interno del mezzo attraversato. L'energia riflessa è pertanto ridotta a causa di un parziale assorbimento della radiazione ad opera del campione; l'informazione relativa alla quantità di energia assorbita è rilevata dal detector, con la conseguente rappresentazione di uno spettro. I materiali generalmente impiegati per la produzione dei cristalli IRE sono caratterizzati da un altissimo indice di rifrazione; tra questi i più comuni sono selenuro di zinco e il germanio (Ge).

5.2.7 Analisi termica differenziale (DSC)

La calorimetria a scansione differenziale (DSC) è una tecnica analitica strumentale che consente di valutare i cambiamenti delle proprietà chimico-fisiche della materia in funzione della temperatura attraverso la rivelazione dei cambiamenti termici associati alle fasi di transizione come cristallizzazione, fusione e transizioni vetrose. Questa tecnica presenta indiscutibili vantaggi tra cui: estrema versatilità, semplicità applicativa, rapidità, scarso o nullo impatto ambientale, utilizzo di campioni tal quali senza necessitare di modifica delle caratteristiche iniziali.

Il calorimetro differenziale a scansione è uno strumento che può essere utilizzato per caratterizzare transizioni del primo ordine (fusione: denaturazione proteica, gelatinizzazione dell'amido, fusione del grasso) o del secondo o pseudo secondo ordine (transizione vetrosa: sistemi a base di granuli di amido o alimenti congelati). Con tale strumento è possibile inoltre studiare la stabilità ossidativa dei grassi, la purezza degli aromi, il procedere di reazioni enzimatiche, la crescita microbica o per sviluppare modelli di previsione della qualità degli alimenti, nonché simulare i trattamenti termici. È stata applicata anche alla cristallizzazione dei grassi e si è rivelata un utile complemento delle tecniche analitiche più tradizionali come la gascromatografia e la gascromatografia accoppiata alla spettrometria di massa.

Il calorimetro a scansione differenziale confronta il passaggio di flusso termico del campione con quello di un materiale di riferimento inerte sia in fase di riscaldamento che di raffreddamento. Dall'analisi in DSC si ottengono dei termogrammi caratterizzati da picchi endotermici e/o esotermici, la cui area è proporzionale all'entalpia assorbita o persa dal campione durante la transizione di fase; questa si verifica durante una specifica scansione di temperatura e dipende dalla composizione e dalle proprietà fisiche del campione in esame.

L'analisi calorimetrica si svolge all'interno di una piccola fornace all'interno del calorimetro, resistente alla corrosione, che si scalda e si raffredda rapidamente. La temperatura della fornace viene misurata e controllata da una resistenza di platino.

Il campione sottoposto ad analisi può essere allo stato liquido, solido, in forma di polvere, di film o di granuli, e deve essere ben distribuito nel porta campione (capsula di alluminio usa e getta) e sempre tenuto sotto costante raffreddamento. Tramite l'unità di controllo, una volta inseriti campione e riferimento nella fornace, si chiude ermeticamente la stessa in modo da isolare l'ambiente di prova dall'esterno.

Viene poi caricato il programma termico (solitamente una rampa lineare di temperatura) e all'interno della fornace contenente il materiale da analizzare viene creata un'atmosfera

inerte con un flusso continuo ed uniforme di azoto. Iniziata la prova, il calore ceduto dalla fornace riscalda sia il campione che il riferimento in egual modo. Durante tutto l'arco dell'esperimento un sistema di termocoppie raccoglie i dati di temperatura. L'elaborazione dei dati consiste nel rilevare i flessi di un grafico che riporta il flusso termico espresso in W/g in funzione della temperatura espressa in °C. La visualizzazione mediante software dei termogrammi permette di calcolare l'entalpia correlata al processo dall'area del picco esotermico o endotermico associato alla transizione di fase e i valori di temperatura iniziale e finale del processo stesso.

5.2.8 Applicazioni della tecnica DSC all'analisi degli oli vegetali

In ambito applicativo nel settore degli oli vegetali si può far riferimento alle attività svolte dal gruppo di ricerca del Dipartimento di Ingegneria Industriale dell'Università di Parma in collaborazione con altri ricercatori Italiani e stranieri, che ha intrapreso a partire dal 2005, uno studio di valutazione sulla applicazione della tecnica DSC a matrici grasse e ad oli di origine vegetale rivolgendo particolare attenzione allo studio della sua applicabilità all'olio di oliva.

Sono stati presi in considerazione diversi ambiti di ricerca tra cui :

- la correlazione tra le proprietà termiche con le analisi relative alla composizione chimica di oli di oliva extravergini monovarietali [16-18] per interpretare le correlazioni tra composizione chimica e parametri qualitativi caratterizzanti oli prodotti da una sola varietà di olivo e proprietà termiche;
- l'individuazione di frodi legate all'aggiunta di oli a minor valore commerciale (olio di nocciola raffinato o olio di girasole ad alto contenuto di acido oleico) in olio extravergine di oliva [19] per una determinazione qualitativa rapida e semplice di questa pratica illecita diffusa nel settore degli oli extravergini di oliva;
- la discriminazione di oli di oliva appartenenti a diverse categorie commerciali sulla base dei diversi termogrammi ottenuti [20];
- l'influenza del riscaldamento a microonde sulle proprietà termiche di differenti categorie commerciali di oli di oliva [21] con la relativa valutazione delle modifiche delle proprietà termiche in funzione del grado di ossidazione raggiunto;
- la valutazione delle variazioni indotte nelle proprietà termiche in raffreddamento dalla conservazione degli oli durante test di invecchiamento in condizioni di ossidazione accelerata per olio di oliva [22] simulando condizioni che meglio ricalcano quelle di un prolungato stoccaggio del prodotto.

Da queste attività di ricerca risulta che le proprietà termiche che si ottengono dai profili di cooling e melting di un olio forniscono importanti informazioni sulle sue caratteristiche qualitative, conservative ed ossidative.

5.2.9 Il naso elettronico

L'identificazione delle sostanze volatili può essere effettuata in modo accurato e preciso attraverso i tradizionali metodi di analisi chimica strumentale, principalmente: la gascromatografia (GC) combinata con la spettrometria di massa (MS). Per contro, tali metodi di analisi sono decisamente costosi, richiedono personale specializzato per il loro utilizzo e raramente possono essere impiegati per monitoraggi di processo o in ambienti diversi dai laboratori. Ciò ha determinato un crescente interesse per nuovi sistemi di analisi che possano fornire informazioni anche parziali sulle miscele di sostanze volatili (ad esempio un controllo di qualità, senza la necessità di determinare l'esatta composizione chimica).

In tale ambito si inseriscono i sensori chimici, dispositivi che convertono una grandezza chimica, generalmente una variazione di concentrazione o pressione parziale di un gas o di una miscela di gas, in un segnale elettrico. Fra i vari tipi di sensori chimici possiamo citare le classi principali, in base al loro principio di funzionamento: *sensori conduttometrici, sensori piezoelettrici, sensori elettrochimici o amperometrici e MOSFET (metal-oxidesemiconductor field-effect-transistor)***.**

Due sono le tipologie di sensori appartenenti alla prima classe: gli ossidi metallici semiconduttori (***MOS***: *metal oxide semiconductor*) ed i polimeri conduttori (***CP***: *conducting polymer*). Entrambi esibiscono una variazione di conducibilità elettrica quando esposti ad una atmosfera contenente ossigeno ed altri gas- La variazione di resistenza del dispositivo si può misurare molto semplicemente applicando una piccola differenza di potenziale ai capi degli elettrodi e sfruttando la legge di Ohm; tali dispositivi hanno infatti un comportamento ohmico all'interno dell'intervallo ±10 V. I sensori a semiconduttore tipicamente disponibili sul mercato hanno come materiale sensibile ossido di stagno (SnO_2), ossido di zinco (ZnO), ossido di titanio (TiO_2), ossido di tungsteno (WO_3).

L'importanza della riproducibilità del dispositivo risulta evidente da quando è stato introdotto il concetto di naso elettronico, come insieme (*serie*) di sensori.

Questi sensori presentano tuttavia alcuni inconvenienti: sono generalmente non selettivi nei confronti di una determinata specie gassosa, mostrano una cross-sensibilità all'umidità, tendono a modificarsi nel tempo (problema della stabilità della risposta o *drift*) rendendo

necessaria una taratura periodica e necessitano di essere riscaldati a temperature fra i 200 ed i 400 °C per poter funzionare. Infatti a temperatura ambiente sarebbero troppo isolanti. Per incrementare la selettività dei singoli sensori sono stati compiuti numerosi tentativi, soprattutto depositando catalizzatori sulla superficie del film in modo da favorire solo le reazioni desiderate. In realtà si può differenziare la risposta con l'utilizzo di catalizzatori ma non si è ancora riusciti a creare un sensore sensibile esclusivamente ad una specie gassosa.

5.3 L'analisi statistica multivariata quale strumento per l'interpretazione dei risultati analitici

Con le denominazioni scienze omiche che si sono diffuse ampliamente negli ultimi anni si indica l'applicazione in diversi campi scientifici dell'approccio fruttuoso della metabolomica. Quest'ultima denominazione indica lo studio sistematico delle esclusive impronte chimiche lasciate da specifici processi cellulari. Il primo vero lavoro scientifico che ha visto l'applicazione della metabolomica risale a più di 40 anni fa [23]. Infatti nel 1971 Pauling pubblico un lavoro [23] in cui l'idea concepita insieme ai suoi collaboratori era che i dati ricchi di informazioni che riflettono lo stato funzionale di un sistema biologico complesso risiede nel modello quantitativo e qualitativo dei metaboliti nei fluidi corporei. Vent'anni dopo questo approccio fu ampiamente applicato per via della più diffusa disponibilità degli strumenti informatici e statistici necessari a "trattare" tali informazioni. Normalmente nelle scienze omiche si utilizzano tecniche separative e spettrometriche e risonanza magnetica nucleare combinate con metodi fisici di analisi accoppiati a tecniche statistiche multivariate (figura 7).

Nella stessa direzione vanno gli studi che prevedono l'applicazione di strumenti fisici accoppiati a metodi di trattamento statistico dei dati, in tali casi inoltre, a differenza dei processi di analisi omica possono essere eliminate o ridotte le manipolazioni ed i pretrattamenti dei campioni che spesso hanno l'obiettivo di estrarre e concentrare alcune specifiche molecole ma che possono rappresentare fonti di errori e di interferenza. L'utilizzo di mezzi e strumenti fisici accoppiati a metodi statistici permettono di abbattere i costi ed i tempi necessari per le analisi classiche ed allo stesso tempo possono fornire un insieme diverso di informazioni che riguardano tanto la qualità come l'autenticità di prodotti.

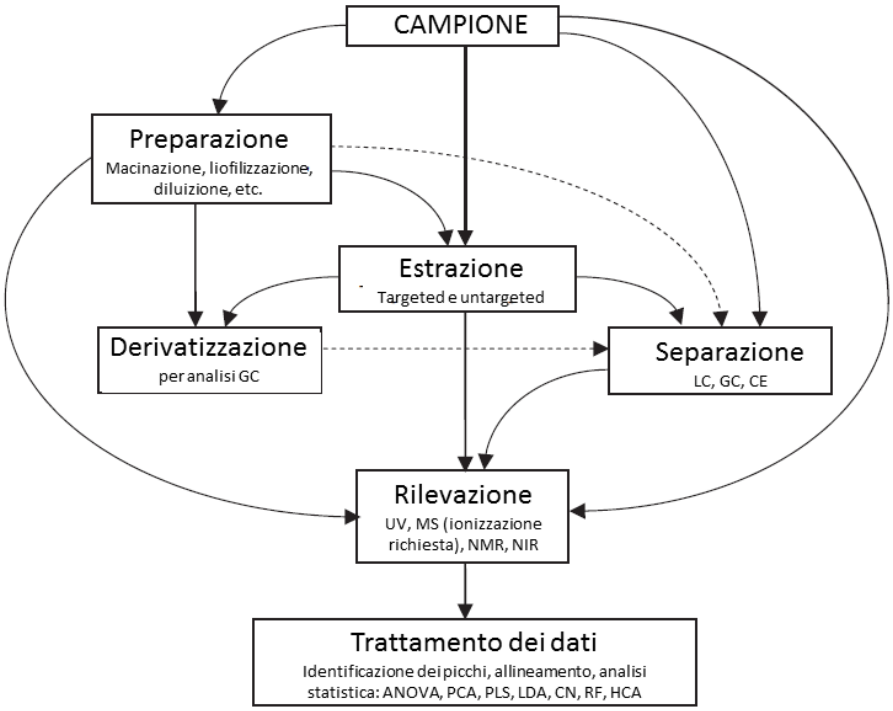

Figura 7
Rappresentazione schematica del processo di analisi omica (rielaborato da [24]).

Per il buon funzionamento di tali metodi è necessaria la costruzione di modelli statistici robusti che utilizzino set di dati correttamente raccolti e rappresentativi del campo di applicazione.

Secondo la definizione dell'enciclopedia Treccani [25] l'analisi statistica multivariata è l'insieme dei metodi statistici e delle tecniche usati nello studio della variazione simultanea di due o più variabili casuali. Date le distribuzioni congiunte di due o più variabili, il sistema più utile per analizzare i dati è quello di rappresentarli sotto forma tabellare o matriciale in cui ciascuna riga rappresenta le caratteristiche osservate sul generico elemento, mentre ciascuna colonna rappresenta la variabilità della stessa caratteristica sui diversi elementi. In particolare, se la matrice dei dati è esprimibile linearmente in funzione di una matrice di variabili indipendenti, si parla di modello lineare multivariato.

Una distinzione essenziale nell'analisi multivariata è se si tratti di analizzare un sistema di covariazione tra gruppi (gli oggetti da esaminare appartengono a più di una popolazione) oppure entro gruppi (gli oggetti appartengono a un'unica popolazione). Le tecniche usate nel primo caso mirano a porre in evidenza un'eventuale disomogeneità dei campioni, e lo

scopo dell'analisi multivariata può essere quello di determinare se i vettori medi delle popolazioni siano o meno uguali (ed è il caso dell'analisi multivariata della varianza), oppure quello di trovare combinazioni lineari delle variabili che massimizzino le differenze tra gruppi preesistenti (analisi discriminante) [25].

Esistono inoltre metodi che consentono di raggruppare gli oggetti a seconda del grado di somiglianza che essi presentano; questi metodi vanno generalmente sotto il nome di *analisi dei clusters*, e lo scopo principale dell'analisi è quello di risolvere una raccolta eterogenea e unica di oggetti in una serie di suddivisioni omogenee al loro interno. Quando gli oggetti da esaminare sono tratti da un'unica popolazione, l'attenzione è rivolta innanzitutto ai modi di semplificare la struttura dei dati. Così, mentre l'analisi dei *clusters* tende a raggruppare quelle variabili che sono altamente correlate, l'analisi dei fattori ha lo scopo di risolvere le correlazioni tra variabili in quelle che si ritengono le loro cause determinanti, cioè di esprimere la covariazione in termini di un certo numero di fattori (inferiori al numero delle variabili) che spieghino una grande parte della varianza e della covarianza delle variabili originarie [25].

6. Conclusioni

Partendo dalle precedenti esperienze di ricerca [26, 27], incentrate sull'impiego della spettroscopia nel medio infrarosso (MIR) a trasformata di Fourier (FT-IR) per valutare il contenuto in acidi grassi, il numero di perossidi ed altri indici ossidativi di oli extravergini di oliva, è stato ideato il lavoro realizzato nell'**Articolo 2**. Nell'**Articolo 2** è stato messo a punto, per la prima volta, un metodo di analisi basato nell'acquisizione di spettri mediante ATR–FTIR e successivo trattamento di dati mediante l'analisi statistica dei minimi quadrati parziali (PLS, partial least squares). In particolare, tale lavoro permette di valutare contemporaneamente il contenuto in acqua, il contenuto totale in composti fenolici e l'attività antiossidante dell'olio che come già discusso sono fortemente connessi con la qualità e con le proprietà salutistiche attribuite agli oli vergini di oliva. L'**Articolo 7**, l'**Articolo 12** e l'**Articolo 13** si inseriscono nello stesso ambito di ricerca, ovvero quello di applicare l'FT-IR per valutare altri parametri qualitativi dell'olio vergine di oliva. In particolare, nell'**Articolo 7** il metodo spettroscopico è stato utilizzato per valutare il contenuto in acidi grassi ossidati (OFA) che rappresentano i prodotti secondari dell'ossidazione lipidica e pertanto connessi con l'invecchiamento e la shelf life degli oli vergini. Nell'**Articolo 7**, è stato realizzato un modello predittivo del contenuto in OFA mediante il trattamento statistico della regressione lineare multipla (MLR) degli spettri acquisiti mediante ATR–FTIR. L'**Articolo 12**, di recente pubblicazione, documenta la messa a punto di un metodo che utilizza l'acquisizione di spettri mediante ATR–FTIR e successivo trattamento statistico PLS per predire il contenuto in alchil esteri in oli d'oliva. Come ampiamente discusso nell'introduzione, il contenuto in alchil esteri è connesso tanto con aspetti qualitativi quanto con la genuinità degli oli vergini di oliva. Anche l'**Articolo 13** utilizza la combinazione degli spettri acquisiti con la tecnica ATR–FTIR ed il successivo trattamento statistico PLS per stabilire in questo caso la qualità della materia prima da cui gli oli sono stati prodotti, ed in particolare il grado di attacco delle olive da *Bactrocera oleae* che rappresenta una delle principali cause del decadimento qualitativo degli oli vergini di oliva. Nell'**Articolo 4** è stata utilizzata sempre la spettroscopia (in questo caso oltre alla MIR anche la Vis-NIR) per acquisire spettri che sono stati trattati con il metodo statistico PLS per realizzare modelli predittivi per stabilire il grado di stress termico subito da oli sottoposti a riscaldamento.

L'**Articolo 5** e l'**Articolo 9** sono in linea alle esperienze precedenti [26, 27] e con l'**Articolo 4** per quanto riguarda la matrice ed i metodi statistici applicati tuttavia la

metodica di analisi applicata in questi lavori è stata l'analisi termica differenziale (DSC). Nelle precedenti esperienze [16-22] di utilizzo di tali metodi sulle sostanze grasse sono stati valutati soltanto alcuni parametri derivanti dalle curve di cooling e melting. Nell'**Articolo 5** e nell'**Articolo 9** invece, sono stati considerati tutti i punti che costituiscono le curve in accordo a quanto avviene nelle scienze omiche. In particolare nell'**Articolo 5** è illustrato il lavoro di ricerca condotto per la realizzazione di un modello predittivo della composizione in acidi grassi utilizzando DSC accoppiata all'analisi PSL. I modelli di regressione realizzati nell'**Articolo 5** sono risultati in grado di predire tanto la composizione in acidi grassi intesa come gruppi (saturi, monoinsaturi e polinsaturi) così come singoli acidi grassi. L'**Articolo 9** documenta il lavoro di ricerca realizzato accoppiando l'analisi DSC all'analisi dei componenti principali (PCA) allo scopo di realizzare modelli in grado di analizzare le caratteristiche di oli sottoposti a stress termici derivanti da riscaldamento per tempi diversi tanto in forno convenzionale come in forno a microonde. Restando sempre sulla matrice olio, l'**Articolo 3** riporta i risultati della ricerca realizzata con l'uso di un naso elettronico a sensori di ossidi metallici (MOS) in combinazione con l'analisi discriminante lineare (LDA) e una rete neurale artificiale (ANN), per prevedere le caratteristiche sensoriali di oli vergini di oliva. Generalmente, e secondo la normativa vigente, tali caratteristiche sono valutate da un panel di assaggiatori esperti. Il lavoro di ricerca descritto ha mostrato la potenzialità del naso elettronico a sensori di ossidi metallici di fornire risultati analoghi a quelli di un panel. L'**Articolo 8** riporta i risultati di un ulteriore studio applicato agli oli vegetali, in questo specifico lavoro di ricerca è stato messo a punto un nuovo metodo di analisi della composizione in acidi grassi mediante cromatografia liquida ad alta prestazione (HPLC) accoppiato al trattamento statistico LDA, al fine di realizzare un modello in grado di predire l'origine botanica di oli vegetali utile per evitare frodi molto frequenti. L'ultimo lavoro sull'olio extravergine di oliva è l'**Articolo 11** in cui viene riportato l'attività di ricerca condotta per la realizzazione di un nuovo processo brevettato per la filtrazione degli oli. In questo caso sono state condotte diverse analisi qualitative per la valutazione dei diversi trattamenti condotti durante la sperimentazione. L'analisi PCA ha permesso di individuare l'effetto esplicato dalle diverse tipologie di filtrazione sulle caratteristiche degli oli, in modo da individuare il sistema più idoneo allo scopo. L'**Articolo 1**, l'**Articolo 6** e l'**Articolo 10** riguardano l'attività di ricerca condotta su dei campioni di formaggio. In particolare, l'**Articolo 1** riporta i risultati delle prove sperimentali condotte per la messa a punto di un metodo di analisi ATR-FTIR accoppiato all'analisi LDA per discriminare i processi di conservazione subiti da diversi formaggi pecorini allo scopo di realizzare un

modello in grado di individuare l'effettiva stagionatura obbligatoria per i formaggi a denominazione di origine Formaggio di Fossa di Sogliano al Rubicone e Talamello. Lo stesso obiettivo è stato conseguito nell'**Articolo 6** mediante l'impiego di un naso elettronico MOS applicando l'analisi statistica ANN. Nell'**Articolo 10** è stato realizzato mediante il trattamento statistico LDA degli spettri acquisiti mediante ATR–FTIR per la creazione di un modello predittivo per attestarne l'originalità di formaggi Parmigiano-Reggiano grattugiati.

I risultati di queste attività di ricerca hanno mostrato l'utilità di analizzare la totalità delle informazioni fornite da diversi strumenti di analisi (ad esempio gli interi spettri o l'intero segnale di un sensore MOS) o di poter combinare i dati ottenuti dalle singole analisi per realizzare modelli statistici predittivi o classificativi utili al fine di stabilire la qualità o genuinità di un olio vegetale o più nello specifico di oli extravergini di oliva così come per quanto attiene all'aspetto origine per alcune categorie di formaggi a pasta dura.
La maggior parte delle metodiche analitiche impiegate in questa attività di tesi hanno il vantaggio di non richiedere solventi nonché di poter essere facilmente automatizzabili e di consentire una semplice interpretazione dei risultati grazie all'utilizzo degli strumenti statistici accoppiati.

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ARTICOLO 1

Lerma-García, M.J., Gori, A., **Cerretani, L.**, Simó-Alfonso, E.F., Caboni, M.F. Classification of Pecorino cheeses produced in Italy according to their ripening time and manufacturing technique using Fourier transform infrared spectroscopy. *Journal of Dairy Science* 93 (10), pp. 4490-4496, 2010 (IF 2,564).

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Classification of Pecorino cheeses produced in Italy according to their ripening time and manufacturing technique using Fourier transform infrared spectroscopy

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ABSTRACT

Fourier-transform infrared spectroscopy, followed by linear discriminant analysis of the spectral data, was used to classify Italian Pecorino cheeses according to their ripening time and manufacturing technique. The Fourier transform infrared spectra of the cheeses were divided into 18 regions and the normalized absorbance peak areas within these regions were used as predictors. Linear discriminant analysis models were constructed to classify Pecorino cheeses according to different ripening stages (hard and semi-hard) or according to their manufacturing technique (fossa and nonfossa cheeses). An excellent resolution was achieved according to both ripening time and manufacturing technique. Also, a final linear discriminant analysis model considering the 3 categories (hard nonfossa, hard fossa, and semi-hard nonfossa) was constructed. A good resolution among the 3 categories was obtained.
Key words: Fourier transform infrared spectroscopy, linear discriminant analysis, Pecorino cheese, ripening time

INTRODUCTION

Several traditional Italian dairy products are made from ewe's milk, particularly ewe's cheeses, which are the predominant food products made from ewe's milk. They are also known with the generic term "Pecorino," indicating all cheeses made only from raw or thermized ewe's milk and mainly produced in the middle and south of Italy according to ancient and unique manufacturing techniques. Some of them are known worldwide because they have the prestigious appellation of "Protected Denomination of Origin" (such as Pecorino Sardo, Pecorino Romano, Pecorino Toscano, Pecorino Siciliano, Fiore Sardo, Canestrato Pugliese, and Fossa cheese from Sogliano al Rubicone and Talamello); however, a large number of Pecorino cheeses exist in Italy that have a geographical origin but do not have the Protected Denomination of Origin appellation. Regarding fossa cheese, a typical feature is seasoning in flask-shaped pits that are dug in the tufa soil of Sogliano al Rubicone (Emilia-Romagna region, Italy). These cheeses are ripened in the pits from 80 to 100 d only after a period of maturation in rooms, which is necessary to achieve a certain degree of consistency. The full ripening takes a minimum of 3 mo to a maximum of 8 mo.

Several factors affect the typicity of cheeses: the geographical area (the fodder/pasture consumed by the animals), the cheese processing, and the degree of ripening. Degree of ripening influences the final development of the sensory properties of the cheese through biochemical events (proteolysis, glycolysis, and lipolysis) that occur during this phase of the cheese processing (Fox et al., 1993; McSweeney and Sousa, 2000).

Several analytical methods are used to evaluate these processes (Luykx and van Ruth, 2008). The utility of these methods has been described in depth in cheese ripening evaluation (Gagnaire et al., 2001; Moatsou et al., 2002; Gorostiza et al., 2004; Poveda et al., 2004; Verdini et al., 2004), even though many cheesemakers still assess the ripening status by a limited number of measurements (e.g., pH and weight) or by visual and tactile examination. Current analytical methods, consisting of chemometric elaborations of physicochemical parameters and secondary proteolysis indices (Contarini et al., 2001; Poveda et al., 2004) or peptide and AA chromatograms (Fallico et al., 2004), are destructive, expensive, and time consuming; in addition, these methods require highly skilled operators. Therefore, there is increasing interest in noninvasive and nondestructive techniques that rapidly estimate the ripening time when required from an easy-to-use and low-cost method. Fourier transform infrared (**FTIR**) spectroscopy is a direct, reliable, and fast method (Karoui et al., 2004) that can be easily applied in fundamental research, in control laboratories, and on-line in the factories to analyze food products. An FTIR spectrometer obtains infrared spectra (4,000 to 700 cm^{-1}) by collecting an in-

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Table 1. Number of samples corresponding to the different geographical origins (city and region of Italy), ripening times, and manufacturing techniques of the Italian Pecorino cheeses employed in this study

	Ripening time and manufacturing technique		
		Hard	
Geographical origin	Semi-hard nonfossa	Fossa	Nonfossa
L'Aquila (Abruzzo)	1	—	1
Potenza (Basilicata)	1	—	1
Avellino (Campania)	1	—	—
Forlì-Cesena (Emilia-Romagna)	—	7	2
Rimini (Emilia-Romagna)	2	1	1
Trieste (Friuli-Venezia-Giulia)	—	—	1
Pesaro-Urbino (Marche)	1	2	2
Nuoro (Sardegna)	1	—	3
Oristano (Sardegna)	1	—	1
Pisa (Toscana)	—	1	2

terferogram of a sample signal with an interferometer, which measures all of infrared frequencies simultaneously. It makes it possible to assign specific bands to specific chemical entities because bands are associated with vibrations of functional groups of the molecules (Coates, 2000). Fourier transform infrared spectroscopy has been used to investigate associated bands of proteins, fats, lactose, and lactic acid in dairy products, and recently they have been applied on ripened cheese to measure their composition (Rodriguez-Saona et al., 2006), sensory and texture parameters (Blazquez et al., 2006), geographic origin (Karoui et al., 2005), and ripening time (Chen et al., 1998; Mazerolles et al., 2001; Downey et al., 2005; Martin-del-Campo et al., 2007). Because no studies have yet been carried out on ewe's milk cheese, the purpose of this investigation was to classify different Italian Pecorino cheeses according to their ripening time and manufacturing technique by attenuated total reflectance (**ATR**) FTIR spectroscopy, dividing the spectra into 18 regions (peaks and shoulders observed) and using linear discriminant analysis (**LDA**) of the spectral data.

MATERIALS AND METHODS

Pecorino Cheeses and Sample Treatment

The study was carried out in 33 Pecorino cheeses produced in 8 different Italian regions (Table 1). Samples were classified as follows: semi-hard nonfossa (ripening times between 2 and 6 mo) and hard fossa and nonfossa (ripening times higher than 6 mo), with fossa and nonfossa being the different manufacturing techniques employed to obtain the cheeses. All the samples were kindly donated by the organizers of the sixth national concourse "Ovillus Aureus, the Gold Pecorino Cheese" (November 28th, 2008, Fano, Italy) reserved to the typical Italian Pecorino cheeses and fossa Pecorino cheeses. The ripening time and manufacturing technique of all the samples were guaranteed by the suppliers.

For FTIR analysis, 3 portions of 15 g of each Pecorino cheese were randomly taken, triturated with mortar and pestle, and mixed. Then, 1g from each portion was removed and measured.

FTIR Spectra

All spectra were acquired using a Tensor 27 FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid interferometer and a DigiTect detector system coupled to an ATR accessory. The ATR was a Pike MIRacle accessory (Pike Technologies, Madison, WI) for the analysis of solids among other materials. This ATR was equipped with a ZnSe reflection crystal and a MIRacle high-pressure clamp (all equipment from Bruker Optics). All analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the range of 4,000 to 700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 software (Bruker Optics).

Each Pecorino cheese was deposited in the ATR surface and pressed with the pressure clamp to obtain a thin cheese layer. Then, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Three spectra were recorded for each portion. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

Data Treatment and Statistical Analysis

Fourier transform infrared spectra were divided into the 18 wavelength regions described in Table 2. Each selected spectral region corresponds to a peak or a shoulder, representing structural or functional group information. For each region, the peak/shoulder area

Table 2. Fourier transform infrared spectral regions selected as predictor variables for statistical data treatment

Identification no.	Range, cm^{-1}	Functional group	Nominal frequency[1]	Mode of vibration
1	3,703–3,323	-N-H -O-H	3,350	Stretching
2	3,323–3,012	-N-H -O-H	3,180	Stretching
3	3,012–2,948	= C-H (*cis*) -C-H (CH_3)	3,006 2,953	Stretching Stretching (asymmetrical)
4	2,948–2,879	-C-H (CH_2)	2,924	Stretching (asymmetrical)
5	2,879–2,729	-C-H (CH_2)	2,853	Stretching (symmetrical)
6	1,807–1,721	-C = O (ester)	1,746	Stretching
7	1,721–1,594	-C = O (acid, amide I)	1,711	Stretching
8	1,594–1,485	-N-H (amide II)	—	Bending
9	1,485–1,433	-C-H (CH_2) -C-H (CH_3)	1,465 1,450	Bending (scissoring) Bending (asymmetrical)
10	1,433–1,375	= C-H (*cis*) -C-H (CH_3)	1,417 1,377	Bending (rocking) Bending (symmetrical)
11	1,375–1,335	O-H	1,359	Bending (in plane)
12	1,335–1,301	Unassigned	1,319	Bending
13	1,301–1,260	C-C(= O)-O (ester)	1,275	Stretching
14	1,260–1,211	-C-O $-CH_2-$	1,238	Stretching Bending
15	1,211–1,139	-C-O $-CH_2-$	1,163	Stretching Bending
16	1,139–1,105	-C-O	1,118	Stretching
17	1,105–1,018	-C-O	1,097 1,033	Stretching
18	1,018–955	-HC = CH- (*trans*)	968	Bending (out of plane)

[1]According to Silverstein et al. (1981), Chen et al. (1998), Rodriguez-Soana et al. (2006), and Lerma-García et al. (2010).

was measured. To reduce the variability associated with the total amount of sample used, and to minimize other sources of variance also affecting the intensity of all the peaks, normalized rather than absolute areas were used. Then, the area of each region was divided by each of the areas of the other 17 regions; in this way, and because any pair of areas should be considered only once, $(18 \times 17)/2 = 153$ normalized variables (predictors) were obtained.

These predictors were used as variables for LDA models, which were constructed using SPSS software (v. 12.0.1, SPSS Inc., Chicago, IL). Linear discriminant analysis, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing Wilks' lambda are obtained (Vandeginste et al., 1998). This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of Wilks' lambda approaching zero are obtained with well-resolved categories, whereas overlapped categories made Wilks' lambda approach 1. Up to $N - 1$ discriminant vectors are constructed by LDA, N being the lowest value for either the number of predictors or the number of categories. The selection of the predictors to be included in the LDA models was performed using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the reduction of Wilks' lambda produced after its inclusion in the model exceeds the entrance threshold of a test of comparison of variances or *F*-test (F_{in}). However, the entrance of a new predictor modifies the significance of those predictors that are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold (F_{out}) is used to decide whether one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The default probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

RESULTS AND DISCUSSION

FTIR Analysis

Fourier transform infrared spectra of the 33 Pecorino cheeses indicated in Table 1 were collected. The spectra of 6 samples, corresponding to 2 semi-hard non-fossa, 2 hard fossa, and 2 hard nonfossa cheeses, are shown in Figure 1. Several differences between the spectra of these cheeses were observed, especially in regions 3,700 to 3,000 and 1,700 to 1,000 cm^{-1}. However, differences in these regions were observed for cheeses corresponding

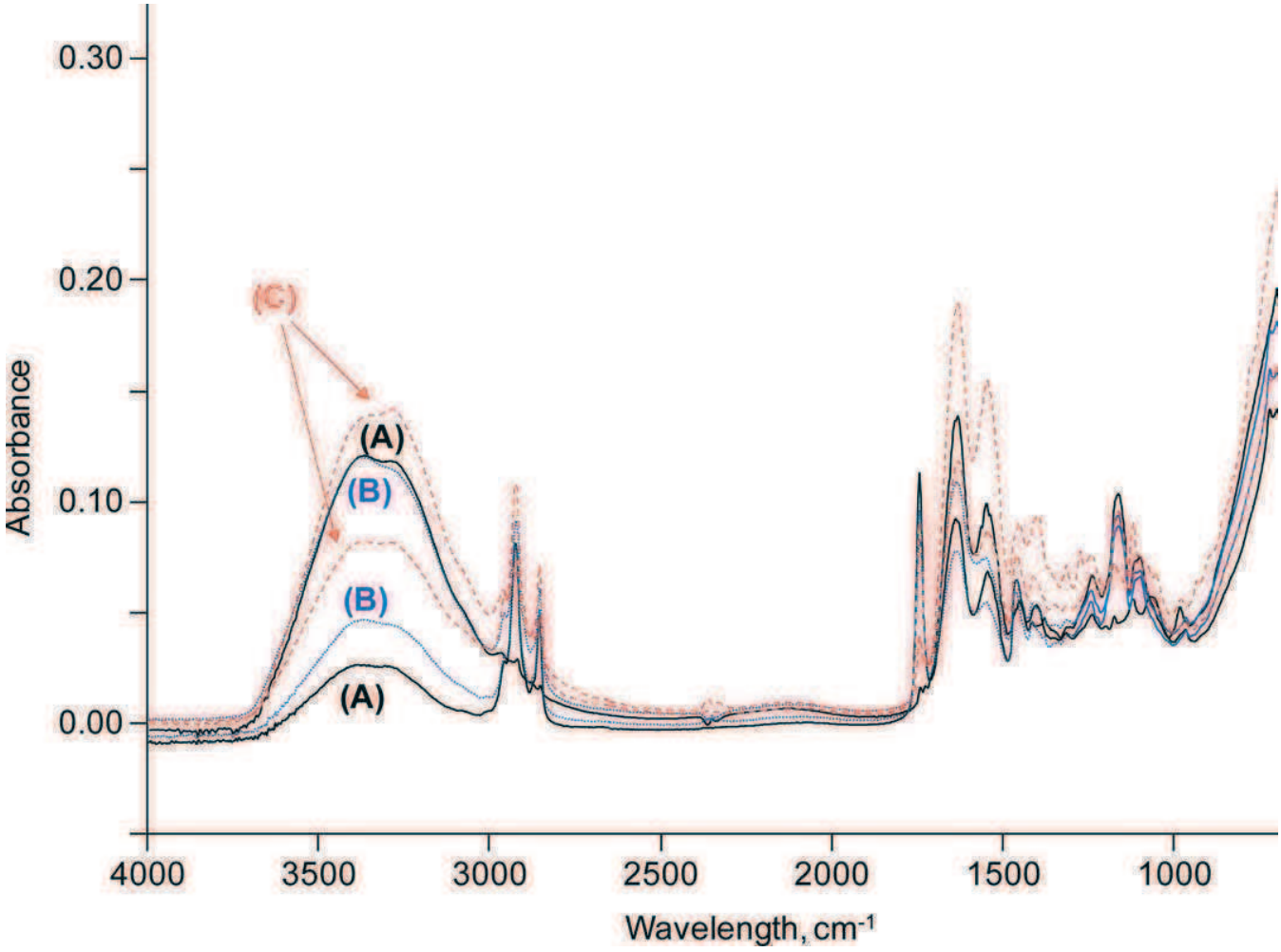

Figure 1. Fourier transform infrared spectra of Pecorino cheese samples of semi-hard (A, solid lines), hard fossa (B, dotted lines), and hard nonfossa (C, dashed lines). Color version available in the online PDF.

to a same category. Then, to enhance all the differences that were not appreciated straight away in the spectra of the cheeses, the peak areas of the 18 selected regions were conveniently handled by multivariate statistical techniques.

Classification of Pecorino Cheeses According to Their Ripening Times

A first LDA model was constructed to classify the samples according to their ripening time (semi-hard and hard). For this purpose, a matrix containing the 99 available objects (which corresponded to the mean of the 3 spectra measured for each portion) and the 153 predictors obtained after the normalization procedure was constructed. This matrix was randomly divided into 2 sets: training and evaluation. The training set comprised 89 objects (19 semi-hard and 70 hard extracts) and the evaluation set comprised 10 objects (5 semi-hard and 5 hard extracts). Another column, containing the 2 categories (semi-hard and hard), was also added to these matrices. An excellent resolution between both categories was achieved with a Wilks' lambda of 0.055. The variables selected by the SPSS stepwise algorithm and the corresponding standardized coefficients of the model are given in Table 3. According to this table, the main infrared (**IR**) regions selected by the algorithm to construct the LDA model corresponded to -N-H (stretching and bending), -O-H (stretching), -C-O (stretching), -C-H (CH_2 and CH_3, bending), -C = O (ester, stretching), and -C-O (stretching). A plot representing the discriminant function against the category was shown in Figure 2. When a leave-one-out validation method was applied to the training set, all the points

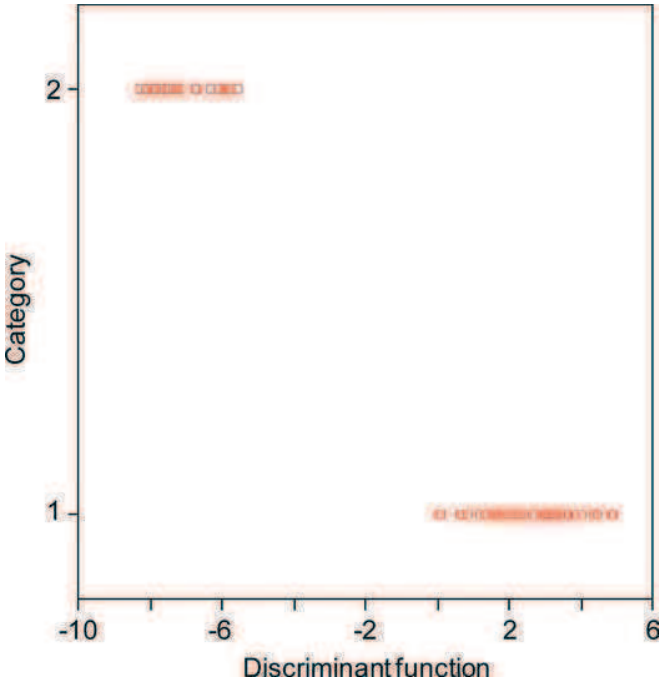

Figure 2. Score plot on the plane of the discriminant function versus the category (1 = semi-hard; 2 = hard) of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their ripening time. Color version available in the online PDF.

were correctly classified. Concerning the evaluation set, all the objects were correctly assigned with an assignment probability higher than 95%.

Classification of Pecorino Cheeses According to Their Manufacturing Technique

Another LDA model was constructed to classify the cheese samples according to their manufacturing technique. In this case, the training set comprised 89 objects (28 fossa and 61 nonfossa) and the evaluation set comprised 10 objects (5 fossa, 5 nonfossa). Also in this case, an excellent resolution between both categories was achieved with a Wilks' lambda of 0.075. The variables selected by the SPSS stepwise algorithm and the corresponding standardized coefficients of the model are given in Table 4. According to this table, the main IR regions selected by the algorithm to construct the LDA model corresponded to -N-H (stretching and bending), -O-H (stretching and bending in plane), -C-O (stretching), and -CH_2- (bending). A plot representing the discriminant function against the category is shown in Figure 3. When a leave-one-out validation method was applied to the training set, all the points were correctly classified. Concerning the evaluation set, all the objects were correctly assigned with an assignment probability higher than 95%.

Table 3. Predictors selected and corresponding standardized coefficients of the first discriminant function ($f1$) of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their ripening time

Predictor[1]	f_1
1/2	3.43
1/8	−19.62
1/10	13.56
1/17	−19.54
2/9	19.85
4/5	−4.77
6/12	23.86
6/17	−10.74
7/13	4.99
8/14	17.66
8/18	6.36
14/16	−3.10
15/16	19.44

[1]Ratios of wavelength regions (Table 2) obtained according to the normalization procedure.

Classification of Pecorino Cheeses According to both Ripening Time and Manufacturing Technique

Finally, a third LDA model was constructed to classify samples according to both ripening time and manufacturing technique. The training matrix comprised 87 objects (20 semi-hard, 29 hard fossa, and 38 hard nonfossa), and the evaluation set comprised 12 objects (4 semi-hard, 4 hard fossa, and 4 hard nonfossa). With this model, a good resolution between the 3 categories was achieved (Wilks' lambda = 0.212). The variables selected by the SPSS stepwise algorithm and the corresponding standardized coefficients of the model showing the predictors with large discriminant capabilities are given in Table 5. According to this table, the main

Table 4. Predictors selected and corresponding standardized coefficients of the first discriminant function ($f1$) of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their manufacturing technique

Predictor[1]	f_1
1/7	−12.79
2/8	24.37
3/8	−12.05
3/18	4.57
4/13	−14.80
5/7	−3.64
5/12	15.54
5/13	16.31
5/14	−19.36
7/8	9.72
7/10	−16.17
8/11	20.40
11/14	8.20
12/16	−9.58
15/16	20.04

[1]Ratios of wavelength regions (Table 2) obtained according to the normalization procedure.

Table 5. Predictors selected and corresponding standardized coefficients of the first and second discriminant functions ($f1$ and $f2$) of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their ripening time and manufacturing technique

Predictor[1]	f_1	f_2
1/2	1.45	−2.26
1/6	1.04	2.33
1/17	−12.67	11.64
2/3	−2.46	0.001
2/15	−2.96	−11.32
2/17	21.68	2.86
3/14	−10.78	−9.28
5/7	−14.48	−4.93
5/9	15.70	7.07
6/11	−11.14	17.66
6/12	16.29	−12.87
7/17	−9.08	−4.46
8/15	15.54	2.45
10/11	3.56	2.56
10/13	7.48	9.45
10/14	0.04	−4.71
11/15	−11.15	0.14
13/17	2.12	1.07
15/16	4.94	−5.51

[1]Ratios of wavelength regions (Table 2) obtained according to the normalization procedure.

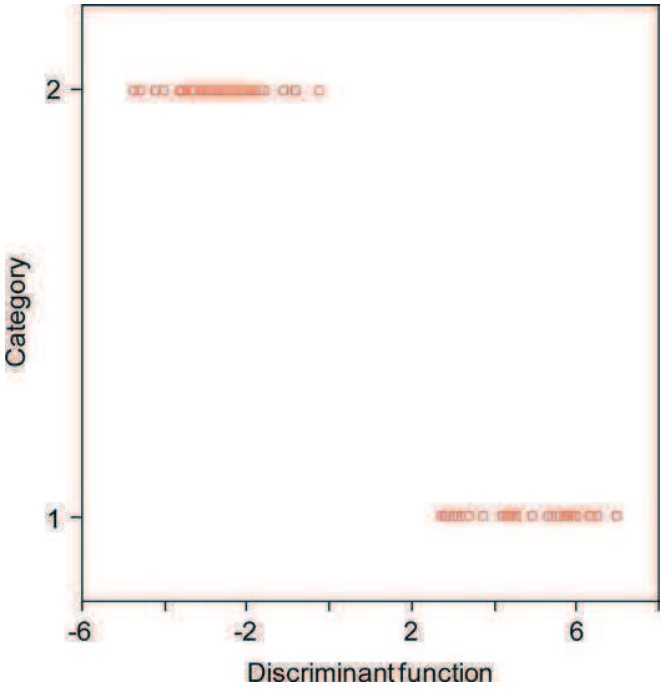

Figure 3. Score plot on the plane of the discriminant function versus the category (1 = fossa; 2 = nonfossa) of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their manufacturing technique. Color version available in the online PDF.

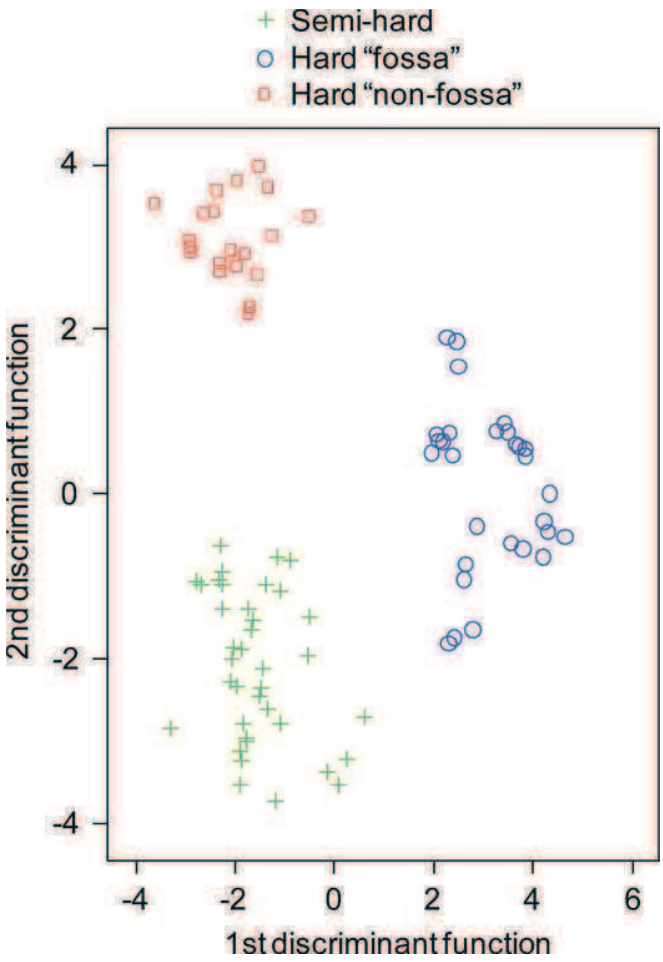

Figure 4. Score plot on the plane of the first and second discriminant functions of the linear discriminant analysis model constructed to classify Pecorino cheeses according to their ripening time and manufacturing technique. Color version available in the online PDF.

IR regions selected by the algorithm to construct the LDA model corresponded to -N-H (stretching), -O-H (stretching and bending), -C-O (stretching), and -C = O (ester, stretching). Figure 4 shows a score plot on the plane of the 2 discriminant functions obtained. When a leave-one-out validation method was applied to the training set, all the points were correctly classified. Concerning the evaluation set, all the objects were correctly assigned with an assignment probability higher than 95%. Thus, the possibility of classifying Pecorino cheeses according to their ripening time, manufacturing technique, or both by using FTIR data has been demonstrated.

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ARTICOLO 2

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Research Article

Rapid FTIR determination of water, phenolics and antioxidant activity of olive oil

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A rapid Fourier transformed infrared (FTIR) attenuated total reflectance (ATR) spectroscopic method was applied to the determination of water content (WC), total phenol amount (TP) and antioxidant activity ($ABTS^{\cdot+}$) of virgin olive oils (VOO) and olive oils. Calibration models were constructed using partial least squares regression. Oil samples with WC ranging from 289 to 1402 mg water/kg oil, with TP from 46 to 877 mg gallic acid/kg oil and with $ABTS^{\cdot+}$ from 0 to 5.7 mmol Trolox/kg oil were considered for chemometric analysis. Better results were obtained when selecting suitable spectral ranges; in particular, from 2260 to 1008 cm^{-1} for WC, from 3610 to 816 cm^{-1} for TP and from 3707 to 1105 cm^{-1} for $ABTS^{\cdot+}$. Satisfactory LOD values by the FTIR-chemometric methods were achieved: 9.4 (mg/kg oil) for WC; 12.5 (mg gallic acid/kg oil) for TP, and 0.76 (mmol Trolox/kg oil) for $ABTS^{\cdot+}$. The evaluation of the applicability of these analytical approaches was tested by use of validation sample sets ($n = 16$ for WC, $n = 11$ for TP and $n = 14$ for ABTS) with nearly quantitative recovery rates (99–114%). The FTIR–ATR method provided results that were comparable to conventional procedures.

Practical applications: The presented method is based on ATR–FTIR in combination with multivariate calibration methodologies and permits a simultaneous evaluation of important quality parameters of VOO (WC, TP and $ABTS^{\cdot+}$). This approach represents an easy and convenient means for monitoring olive oil quality with the advantage of ease of operation, speed, no sample pretreatment and no consumption of solvents. The data obtained with this method are comparable to those obtained using the official reference method. Therefore, the technique is highly plausible as an alternative to the standard procedure for routine analysis or control at-line of production processes.

Keywords: Antioxidant / FTIR / Olive oil / Partial least squares model / Water

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Abbreviations: **ABTS$^{\cdot+}$**, antioxidant activity; **ATR,** attenuated total reflectance; **DEO,** mildly deodorized olive oils; **EJRC,** ellipse of joint region confidence; **FTIR,** Fourier transformed infrared; **OO,** olive oil; **PLS,** partial least squares; **REC,** relative error in calibration; **RMSD,** root-mean-square deviation; **TP,** total phenol content; **VOO,** virgin olive oil; **WC,** water content

1 Introduction

Among edible oils, virgin olive oil (VOO) has nutritional, storage and sensory characteristics that make it a unique and basic ingredient of the Mediterranean diet. These properties are attributed not only to the fatty acid composition (high level of oleic acid), which is not significantly different from refined olive oil, but especially to the high content of phenolic compounds, which are reduced by the refining process (in particular during the bleaching and deodorization phases). Unfortunately, a low phenolic content is also seen in many

VOO currently sold a low prices in supermarkets and discount stores, which are most likely illegal blends of VOO and mildly deodorized olive oils (DEO) [1, 2].

Phenolic compounds, acting as natural antioxidants, increase the resistance of oil to storage and heating [3, 4]. Moreover, phenols are the main contributors to the typical tastes of VOO (bitter and pungent attributes), and may also contribute to the prevention of several human diseases [3].

It is well known that phenolic compounds can act as radical scavengers or chain breakers decreasing the total rate of lipid oxidation and in particular the phenols with a catechol moiety, known as *o*-diphenols, are particularly effective antioxidants [5]. The antioxidant activity ($ABTS^{\cdot+}$) of phenolic fraction is measured by different tests, and the $ABTS^{\cdot+}$ radical scavenging test has been largely utilized [6–12].

Among the minor compounds of olive oil, water has been recently taken into consideration. In fact, olive oil also contains microdrops of water that are dispersed in the lipid phase and stabilized by the aggregation–dissolution of a group of polar, water-soluble and/or water-compatible substances such as mineral salts, free acids, diglycerides, phospholipids, alcoholic and phenolic substances. The water content (WC) in edible olive oils varies from 300 to 2000 mg/kg of oil, and is closely affected by several factors: extraction technologies (continuous *versus* traditional systems), technological variables used to process olives [13], addition of extraction coadjuvants, filtration procedures and storage conditions [14]. Therefore, because of the capacity of water to dissolve phenolic compounds and other small or medium-sized molecules having a low affinity to oily phase, water contributes directly to the taste perception of oil bitterness and pungency as well as indirectly to its oxidation stability [14].

In particular, VOO is characterized by an average WC between 1000 and 1200 mg/kg of oil, whereas due to the refining processes which decrease the WC [13], olive oils and olive pomace oils generally have $<$500 mg/kg of oil. The amount of water has also been proposed [13] as an analytical parameter to detect 'mild deodorization'. In fact, this practice is employed to reduce or eliminate small molecules such as volatile and polar compounds, which are responsible for off-flavours of olive oil. This effect is due to a sort of molecular distillation under vacuum or in a stream of nitrogen at a low temperature or by absorption mechanisms on high polarity powders or filtering membranes. As these treatments alter the compositional balance of polar minor compounds in the oil, they affect also the stability of microemulsified water, which inevitably decreases. Hence, samples of mildly deodorized oil or blends of VOO and mildly deodorized oil are expected to have $<$700 mg/kg of oil [13].

The determination of the WC in edible oils has been commonly carried out by the Karl Fischer titration method [13, 15]. In addition, spectroscopic Fourier transformed infrared (FTIR) coupled with chemometric methods have been successfully used to detect olive oil adulteration [16–18] and freshness [19]. The chemometric algorithm partial least squares (PLS) has been repeatedly and extensively used to obtain different quality parameters of edible oils [20–25]. In particular, FTIR–PLS has been recently applied to the evaluation of the fatty acid composition and other quality parameters of VOO [26] and also to predict the total antioxidant capacity of the red wine samples [27].

PLS is a factorial multivariate calibration method that decomposes spectral data into loadings and scores, building the corresponding calibration models from these new variables [28]. This method requires that the analytes comply with Beer's Law in order for the property to be measured.

The aim of the present work was to develop a new application of the FTIR–ATR–PLS association as a rapid, inexpensive and nondestructive tool able to determine very important parameters linked to the quality of olive oils as the water and phenol content and antioxidant activities. This approach represents an easy and convenient method for monitoring olive oil quality with the advantages of ease of operation, high sample turnover and no sample pretreatment.

2 Materials and methods

2.1 Samples

The 47 VOO and 7 olive oil (OO) samples used in this study were purchased in farms and markets in Italy. Samples were stored in dark bottles without headspace at room temperature before analysis.

All experiments and calculations were done in triplicate.

2.2 FTIR spectra

The FTIR spectra were acquired on a Tensor 27^{TM} FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid^{TM} interferometer and a DigiTect^{TM} detector system coupled to an attenuated total reflectance (ATR) accessory. The ATR accessory (Specac Inc., Woodstock, GA, USA) was equipped with a ZnSe 11 reflection crystal. Analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 (Bruker Optics) software. For each sample (2 mL uniformly spread throughout the crystal surface), the absorbance spectrum was collected against a background, obtained with a dry and empty ATR cell. Three spectra per sample were recorded. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

2.3 Determination of water content in virgin olive oil

The WC was analyzed with a TitroMatic 1S instrument (Crison Instruments, S. A.; Alella, Barcelona, Spain). This

measurement uses a Karl-Fischer titration based on a bivoltametric indication (2-electrode potentiometry). A solution of chloroform/hydranal solvent oil (a methanolic solvent) 2:1 v/v was used to dissolve the sample and hydranal-titran 2 was used as a titrating reagent (hydranal-titran 2 and hydranal-solvent oil were from Riedel-deHaën, Seelze, Germany). Each sample was introduced three times and the quantity of the sample was measured using the back weighting technique. The sample was dissolved in a solution of chloroform:hydranal-solvent oil, and the titrating reagent was added until the equivalence point was reached. The quantity of water was expressed as mg water/kg of oil (mean of $n = 3$).

2.4 Extraction of polar phenolic fraction

Phenolic compounds were extracted from VOO and OO by a liquid–liquid extraction method according to Pirisi *et al.* [29]. The dry extracts were dissolved in 0.5 mL of a methanol/water (50:50, v/v) solution and filtered through a 0.2 μm syringe filter (Whatman Inc., Clinton, NJ, USA). Extracts were frozen and stored at −43 °C.

2.5 Spectrophotometric determination of total phenol content

The total phenol (TP) content of the extracts was measured using the Singleton and Rossi [30] method with slight modifications. Phenolic extracts were analyzed spectrophotometrically with Folin–Ciocalteu reagent and absorbance was determined at 750 nm. Total phenols were quantified using a gallic acid calibration curve ($r^2 = 0.994$). The results were expressed as mg gallic acid/kg of oil.

2.6 The $ABTS^{\cdot+}$ assay

A stable stock solution of $ABTS^{\cdot+}$ was produced by reacting a 7 mmol/L aqueous solution of ABTS (Sigma, ST. Louis, MO, USA) with 2.45 mmol/L potassium persulphate (Sigma) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Before use, the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm at 30°C. Next, 1 mL of this $ABTS^{\cdot+}$ solution was added to 0.01 mL of extract and the decrease in absorbance was recorded for 10 min. Absorbance values were corrected for radical decay using a blank solution (0.01 mL of methanol/water 50:50, v/v). Measurements were made in triplicate and the $ABTS^{\cdot+}$ was calculated as mmol Trolox equivalent for kg of oil ($r^2 = 0.9830$) [31].

2.7 Statistical analysis

Data were exported in ASCII compatible OPUS 6.0 format with the assistance of an OPUS macro script and processed employing MVC1 routines [32] written for Matlab (Mathworks Inc., Natick, MA, USA).

PLS models were computed for each analytical parameter with the respective training set samples. A moving-window strategy was also executed with the MVC1 program, setting the minimum window width to 50 sensors.

3 Results and discussion

3.1 Water content, total phenol and antioxidant activity of virgin olive oil and olive oil samples

Forty-seven samples of both VOO and OO (this category of oils is obtained by blending VOO with refined olive oil so that OO are normally characterized by a lower phenolic compound and WC) were analyzed with the aim of obtaining a wide range of variability in the selected parameters. In fact, as shown in Fig. 1, WC ranged from 289 to 1402 mg water/kg oil, TP from 46 to 877 mg gallic acid/kg oil and $ABTS^{\cdot+}$ from 0 to 5.7 mmol Trolox/kg oil. These wide intervals were particularly suited to build a robust calibration model for the FTIR method and thus a challenging validation set.

The TP of VOO, evaluated by the Folin–Ciocalteu test, may range between 40 and 900 mg gallic acid/kg of oil. Nevertheless, a higher concentration (up to 1000 mg gallic acid/kg) has also been reported in several olive oils [33, 34]. As reported by many authors [6–10, 35], the $ABTS^{\cdot+}$ of olive oil, measured by the ABTS radical scavenging test, is highly linear correlated to the TP determined by spectrophotometry, which confirms the important role of phenolic compounds (in particular *o*-diphenols) in protection from autooxidation.

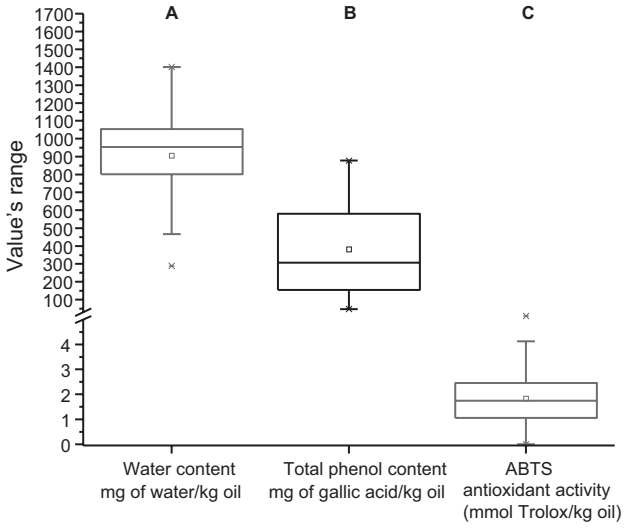

Figure 1. Box and whiskers plot showing the distribution of (A) water content, (B) total phenol content and (C) $ABTS^{\cdot+}$ antioxidant activity in VOO and OO samples.

Both agronomic factors (olive cultivar, place of origin, olive ripening stage, agronomic techniques adopted) and the technological parameters used to process olives (extraction methods and storage conditions) significantly affect the TP as well as the $ABTS^{\cdot+}$ and WC in olive oil so that they vary from very low amounts to high values [6, 13]. For example, VOOs obtained by different extraction techniques from healthy and medium ripe olives coming from different Sardinian cultivars and grown in different geographical areas have a TP amount ranging from 108 to 441 mg gallic acid/kg oil, and an $ABTS^{\cdot+}$ ranging from 1.35 to 2.45 mmol Trolox/kg oil [7]. Moreover, some olive cultivars as Coratina or Nostrana di Brisighella may contain a very high content of phenols (>500 mg gallic acid/kg oil), and many other VOOs produced by unripe olives are characterized by elevated TP and $ABTS^{\cdot+}$ values [3, 13]. In contrast, VOOs obtained from ripe olives or from specific olive cultivars may generally be characterized by a moderate phenolic content (*e.g.* <100–200 mg gallic acid/kg oil), and consequently by poor antioxidant capacity [3, 7, 36].

Technological choices, such as the kind of extraction and filtration system, may have a more direct influence on the content of water remaining in the VOO and, therefore, the antioxidant power in terms of the TP and the $ABTS^{\cdot+}$. Generally, VOO produced by two-phase plants and filtered contain a lower amount of water than those obtained by a traditional system and veiled, *e.g.* 800–900 mg compared to 1100–1300 mg water/kg oil [13, 37, 38].

Additionally, the refining process applied to produce refined oils in OO causes a severe depletion of water and phenolic molecules, and thus a decrease of the antioxidant power, reaching values less than 500 mg water/kg of oil (WC) and 100 mg gallic acid/kg of oil (TP). As for the $ABTS^{\cdot+}$, it can be less than 0.70 mmol Trolox/kg oil [11, 13]. For example, Pellegrini and coworkers [11, 35] studied the TP and the $ABTS^{\cdot+}$ of several commercial olive oils: commercial VOO showed TP and $ABTS^{\cdot+}$ values ranging from 73 to 265 mg gallic acid/kg oil and from 1.53 to 2.69 mmol Trolox/kg oil, respectively. However, the antioxidant power was lower in samples of OO. In fact, the TP values ranged from 14 to 30 mg gallic acid/kg oil, and the $ABTS^{\cdot+}$ of OO ranged from 0.72 to 1.06 mmol Trolox/kg oil.

Mildly deodorized olive oils commonly show physicochemical characteristics similar to those of genuine VOO, although their antioxidant power is highly decreased. In fact, some studies have reported that the WC of blends of VOO and DEO may be less than 700 mg/kg oil, while the TP content may range from 100 to 250 mg gallic acid/kg oil with a $ABTS^{\cdot+}$ less than 0.70 mmol Trolox/kg oil [2]. Domestic heating treatments such as frying, boiling and conventional and microwave heating can also affect the antioxidant properties of commercial olive oils, strongly inducing a high depletion of the TP and, therefore, a decrease of the $ABTS^{\cdot+}$ as a result of the different extent of radical formation and different thermal stability of phenolic compounds [2, 11]. Finally, the storage conditions (storage time and temperature) can significantly decrease the antioxidant power of VOO. A reduction of the TP to values less than 100–200 mg gallic acid/kg oil, and therefore a loss of the antioxidant power was observed after production up to 1 year with more remarkable losses in the last 6 months of storage [8]. With regard to the storage temperature, sudden changes in the temperature may have a significant effect both on the TP and the $ABTS^{\cdot+}$. Although a low storage temperature may slow radical formation, it can still destabilize microdrops of water in which phenols are dissolved, so that the antioxidant power will decrease [6, 39, 40].

3.2 PLS models construction

In order to predict the WC, TP and $ABTS^{\cdot+}$ in VOO, three multivariate calibration models were built by the PLS regression algorithm, using digitalized spectral data. As reported in Fig. 2, the optimal number was obtained by Haaland and Thomas statistical criterion ($a = 0.75$) [41] using Predicted REsidual Sums of Squares (PRESS) (Eq. (1)). PRESS is the sum of squares of the differences between predicted ($\hat{C}_i$) and real values of analyte concentrations (C_i); thus, it is a measure of the predicting ability of the calibration model

$$\mathrm{PRESS} = \left[\sum_{i=1}^{N} (\hat{C}_i - C_i)^2\right]^{0.5} \tag{1}$$

The optimal spectral intervals were obtained by a minimum PRESS search employing the moving window strategy with the 'leave one out' cross-validation procedure (Fig. 3). This procedure is known to enhance the performance of the method [42]. The 'leave one out' cross-validation technique systematically generates PLS validation models by excluding one by one each sample from the dataset and then predicting the value for the omitted sample. After this is accomplished for every sample in the dataset, a PRESS for the PLS cross-validated model is calculated. Additionally, good values of statistical parameters, such as the root-mean-square deviation of calibration (RMSD, Eq. (2)) and the relative error in calibration (REC (%), Eq. (3)) were found (Table 1)

$$\mathrm{RMSD} = \left[\frac{\mathrm{PRESS}}{N}\right]^{0.5} \tag{2}$$

$$\mathrm{REC}(\%) = 100\,\frac{\mathrm{RMSD}}{C_{\mathrm{mean}}} \tag{3}$$

As shown in Table 1, the spectral range selection permitted an increase of the R^2 value for WC and TP, while no increment in the $ABTS^{\cdot+}$ evaluation was observed for R^2. The lower value of R^2 for $ABTS^{\cdot+}$ prediction could be explained considering that this test is based on a kinetic measure having a higher intrinsic variation. In the same

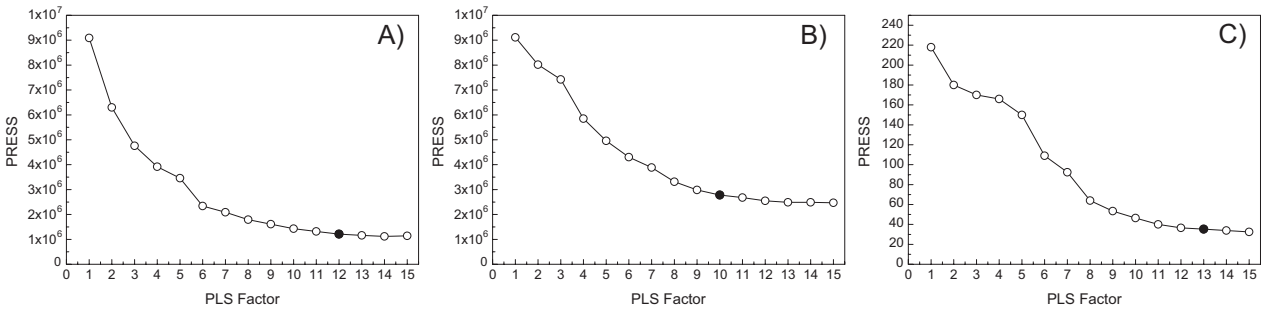

Figure 2. PRESS value *versus* PLS factors for PLS models of (A) water content [(mg/kg oil)^2], (B) total phenol content [(mg gallic acid/kg oil)^2] and (C) ABTS$^{\cdot+}$ antioxidant activity [(mmol Trolox/kg oil)^2], optimization of calibration parameters.

way, the selection of spectral range caused an improvement in mean recoveries, all between 99 and 114% (Table 1). Finally, marked reduction of standard deviation values for TP (81–29%) and ABTS$^{\cdot+}$ (77–33%) models was obtained.

For WC, the optimized model (Fig. 4A) exhibited very good performance in terms of actual *versus* FTIR–PLS predicted values in both the calibration and validation sets of samples. In addition, for total phenol evaluation, the optimized model (Fig. 4B) showed good results in terms of actual *versus* predicted values in both the calibration and validation sets of samples but with an acceptable dispersion in the prediction. On the other hand, ABTS$^{\cdot+}$ estimation model (Fig. 4C) exhibited good performance for the calibration samples but a high dispersion was evident for validation set. Figure 4D–F reports the ellipse of joint region confidence (EJRC) test for the three models. The three EJRC tests evidence the absence of systematic errors because the intercept is closed to 0 and in addition the absence of bias errors in the prediction due to the slope is near to 1.

Moreover, satisfactory LOD values for the three analytical parameters were achieved by the FTIR-chemometric methods: 9.4 (mg/kg oil) for WC; 12.5 (mg gallic acid/kg oil) for TP, and 0.76 (mmol Trolox/kg oil) for ABTS$^{\cdot+}$. In fact, considering the data intervals (Fig. 1) registered for WC (from 289 to 1402 mg water/kg oil), TP (from 46 to 877 mg gallic acid/kg oil) and ABTS$^{\cdot+}$ (from 0 to 5.7 mmol Trolox/kg oil), the calculated LOD values permit to analyze both VOO and OO samples.

4 Conclusions

The results of chemometrically assisted FTIR analysis were statistically similar to those obtained by official and traditional procedures in terms of analytical performance. Moreover, the ATR–FTIR–PLS method developed herein was faster; the complete determination takes only a few minutes compared to the long time required for both the WC analysis (approximately 30 min) by titrimetric analysis and TP and ABTS$^{\cdot+}$ spectrophotometric determinations (several hours taking into consideration phenol extraction, reactive preparation and the long wait for the complete reaction). Therefore, the proposed spectroscopic method can be a highly convenient alternative in terms of time and solvent savings for routine analysis of a large number of VOO samples, especially for high throughput determinations

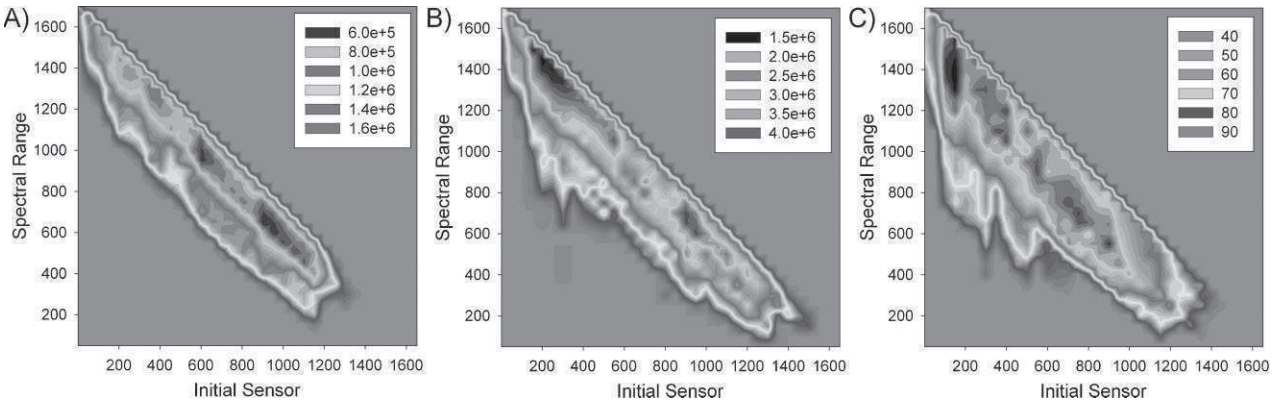

Figure 3. PRESS surface in the spectral range of calibration (sensors) for PLS models of (A) water content [(mg/kg oil)^2], (B) total phenol content [(mg gallic acid/kg oil)^2] and (C) ABTS$^{\cdot+}$ antioxidant activity [(mmol Trolox/kg oil)^2], optimization of calibration parameters.

Table 1. Calibration and validation parameters of the FTIR–ATR–PLS determination for water content (WC, expressed as mg water/kg oil), total phenol content (TP, expressed as mg gallic acid/kg oil) and ABTS$^{\cdot+}$ antioxidant activity (ABTS$^{\cdot+}$, expressed as mmol Trolox/kg oil)

Parameter	WC		TP		ABTS$^{\cdot+}$	
Statistical summary						
Linear range	289–1402		46–877		0.021–5.660	
Number of spectra used in calibration	146		116		145	
Number of spectra used in validation	16		11		14	
Spectral range (cm^{-1})	2260–1008	Full	3610–816	Full	3707–1105	Full
PLS factors	12	12	10	10	13	13
RMSD (conc. units)	78	93	85	120	0.49	0.58
REC (%)	8.6	10.3	22.4	31.5	26.5	31.7
R^2	0.89	0.84	0.87	0.74	0.63	0.67
Mean recovery of validation set (%)	99	103	114	113	98	131
Standard deviation of validation set (%)	8	11	29	81	33	77
Figures of merit						
Sensitivity (10^{-6}, conc units)	7.00	28.0	12.0	22.0	190	360
Analytical sensitivity	0.03	0.024	0.026	0.019	4.1	3.1
Selectivity based on total signal	0.003	0.011	0.003	0.005	0.002	0.004
Mean spectral residue	0.00019	0.001	0.00045	0.001	0.00046	0.001

Parameter improvement by spectral range selection.
RMSD, root mean square deviation; REC, relative error of calibration.

during industrial processing with slight sacrifice of accuracy. This method can also allow in-process optimization of technological parameters (*i.e.* malaxation time and temperature control) of VOO production based on water and phenol content. Furthermore, the procedures permit high sample throughput and are eco-friendly compared to previously reported alternatives, since no sample pretreatment is required and virtually no solvent waste is produced.

The authors have declared no conflict of interest.

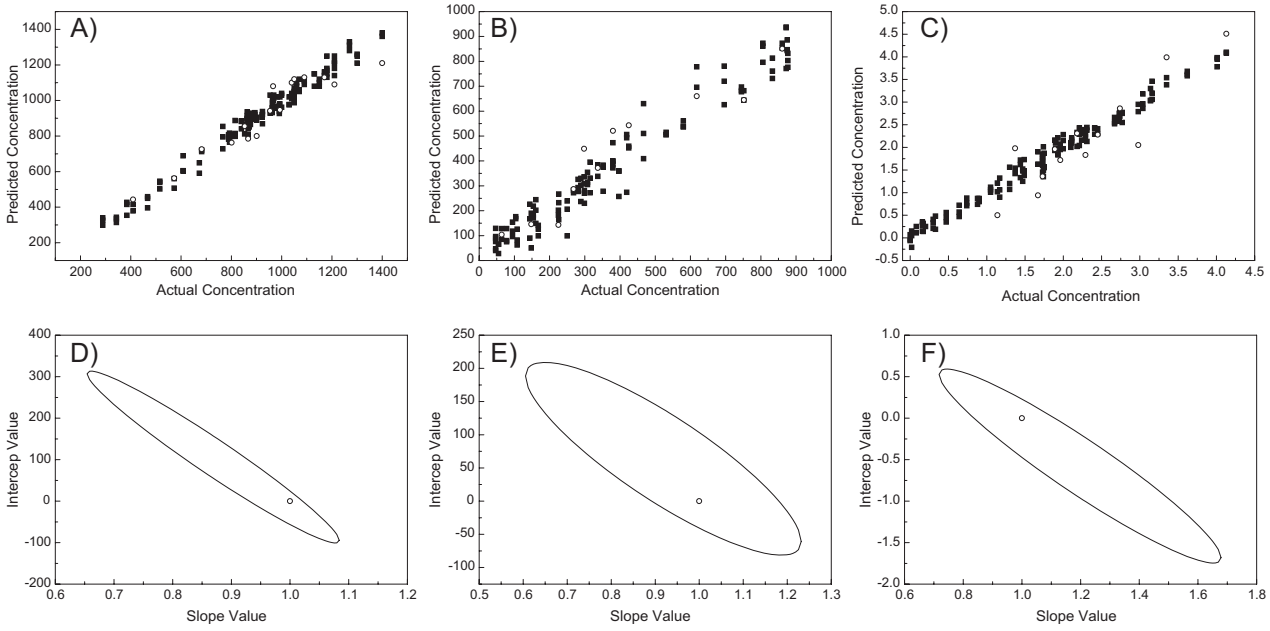

Figure 4. Actual *versus* FTIR–PLS predicted values in the calibration (■) and validation (○) sets for (A) water content (mg/kg oil), (B) total phenol content (mg gallic acid/kg oil) and (C) ABTS$^{\cdot+}$ antioxidant activity (mmol Trolox/kg oil). Ellipse of joint region confidence (EJRC) for slope and intercept of actual *versus* predicted values curve in the validation set (statistical level of significance used is 95%), for (D) water content, (E) total phenol content and (F) ABTS$^{\cdot+}$antioxidant activity.

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ARTICOLO 3

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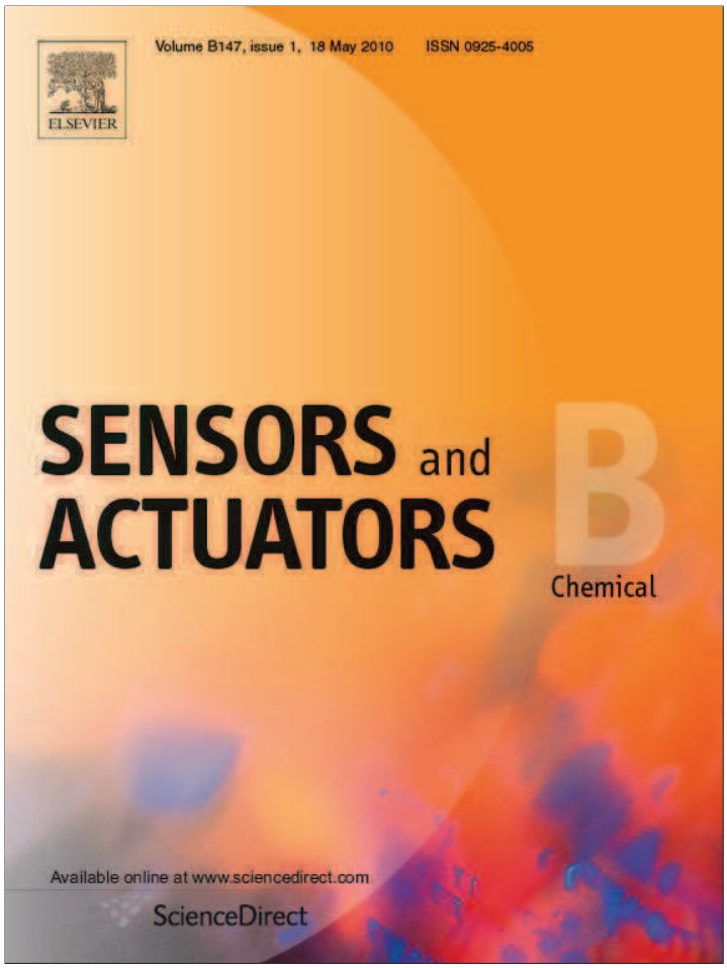

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Use of electronic nose to determine defect percentage in oils. Comparison with sensory panel results

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ABSTRACT

An electronic nose based on an array of 6 metal oxide semiconductor sensors was used, jointly with linear discriminant analysis (LDA) and artificial neural network (ANN) method, to classify oils containing the five typical virgin olive oil (VOO) sensory defects (fusty, mouldy, muddy, rancid and winey). For this purpose, these defects, available as single standards of the International Olive Council, were added to refined sunflower oil. According to the LDA models and the ANN method, the defected samples were correctly classified. On the other hand, the electronic nose data was used to predict the defect percentage added to sunflower oil using multiple linear regression models. All the models were able to predict the defect percentage with average prediction errors below 0.90%. Then, the develop is a useful tool to work in parallel to panellists, for realizing a rapid screening of large set of samples with the aim of discriminating defective oils.

1. Introduction

Food industry, especially dairy industry, has been one of the earliest users of sensory analysis, which is an extremely useful tool for flavour researchers [1]. There are two major types of sensory analysis: affective and analytical. Affective sensory tests are based on consumers and their perceptions of acceptability, and are important to the food industry because they explain the role of flavour, texture, and appearance in influencing consumer acceptability. These types of techniques can only measure what untrained consumers think; they tend to suffer from extensive person-to-person variability. Therefore, polling a large number of consumers (>50) is typically done to improve the statistical validity of the information obtained [1]. On the other hand, analytical sensory techniques are based on trained panelists. Discriminatory tests (difference and threshold), as well as descriptive sensory analysis, are perhaps the most powerful sensory tools. Analytical techniques are well suited for both identifying flavours in a product and discriminating sensory properties between products [1].

The most important phase of olive oil sensory analysis is represented by its aroma. Aroma is a very complex sensation, being identified more than 7100 volatile compounds in foods overall [2,3]. Each volatile compound may *potentially* contribute to aroma perception, depending upon their concentrations and sensory thresholds. The definition of sensory threshold was introduced in sensory analysis as a threshold in which any sensation was perceived. Regarding olive oil sensory analysis, the olfactory sensory threshold of a panel with respect to some defects is evaluated to habilitate the panel as official, recognized by the International Olive Council (IOC) or by the Ministry of the different European Countries. On the other hand, the sensory analysis is one of the most important tools useful to classify the virgin olive oils (VOOs) in different commercial categories (extra virgin, virgin and lampante), which is mainly evaluated with the presence of sensory defects [4]. The most frequent off-flavours of VOO are grouped into five main defects: fusty, muddy, mouldy, winey and rancid. Several works in literature have focused the correlation between defects perceived by a trained panel in VOOs and the presence of specific volatile compounds in the head-space of these samples. Morales et al. [5] have studied VOOs differently defected by dynamic headspace high-resolution gas chromatography coupled with mass spectrometry detection and olfactometry, identifying the volatile compounds mainly responsible for the off-flavours. Considering the ratio between the volatile concentration and its odour threshold in oil the mouldy defect resulted strictly related to the presence of some C_8 compounds produced by specific mould enzymes as 1-octen-3-one and 1-octen-3-ol. On the other hand, the winey defect, due to sugar fermentation, was well described by acetic acid and ethyl acetate whereas

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the fusty unpleasant odour was linked with some branched C_5 components as 3-methyl butan-1-ol as a consequence of an anomalous aminoacid degradation; with regard to rancid sensory defect several saturated and unsaturated aldehydes, as nonanal and *E*-2-heptenal respectively, have been detected as characteristic compounds.

The human being is exceptionally sensitive to some volatiles (such as 2-isobutyl-3-methoxypyrazine, which has an odour detection threshold in water of 0.002 ppb [6] and 0.015 ppb in wine [7], and 1-octen-3-ol, with a detection threshold of 1 ppb in oil) [5], but insensitive to many other volatiles (e.g., ethanol has an odour threshold in water and in oil of 100,000 ppb and 30,000 ppb respectively, and a taste threshold of 52,000 ppb in water) [5,6]. A person's ability to detect odours is also influenced by many other factors such as genetic variability, olfactory fatigue, and naturally occurring and unpredictable factors such as temperature and humidity. The complexity of food aromas and sensitivity required, plus the fact that the olfactory system must be able to respond to unknown odorants (it cannot be learned response), make this a most complex phenomenon. For this reason, considerable interest exists in the development of instrumental techniques, non-invasive and non-destructive, in order to make more objective, faster and less expensive the assessments of olive oil sensory quality [8].

Recently, metal oxide semiconductor (MOS) sensors have been applied in VOO aroma control to detect a variety of sensory defects [9–14] and to authenticate VOOs according to varietal or geographical origin of olives [15]. On the other hand, MOS sensors have a low cost and can work on-line without sample pretreatment [16,17]. These sensors do not provide a quali-quantitative analysis of volatile compounds of the samples, but responds to the whole set of volatiles in a unique digital pattern. These patterns are a signature of the particular set of aromatic compounds such as these should determine a specific olfactory perception [18].

Aparicio et al. [9] have used MOS sensors to detect the rancid defect in VOOs, using the information on volatile compounds responsible for rancidity and the sensory evaluation of the samples by assessors for explaining the mathematical selection of sensors. The same studies have been also performed by Garcia-Gonzalez et al. [11] for the detection of vinegary defect. More recently, Cimato et al. [19] have studied 12 monovarietal extra VOOs from Tuscany by means of three different methodological approaches: metal oxide sol–gel thin films based electronic nose and multivariate data analysis (PCA), headspace-solid phase micro-extraction/gas chromatography/mass spectrometry (HS-SPME/GC/MS) and sensory analysis with the aim to discriminate the different samples. Authors evidenced that HS-SPME/GC/MS was possible to obtain the chemical map of the different samples, while with electronic nose samples were separated in clusters.

More recently, Lopez-Feria et al. [20] have developed a fast method based on the direct coupling of HS-MS without chromatographic separation and multivariate tools (SIMCA and PLS) to determine the presence of negative attributes and to classify VOO. Authors analyzed a training set composed by refined olive samples spiked at different levels (from 20 to 100%) with standard defects whereas the prediction set was made up of several unknown samples belonging to different VOO classes. Despite the good results, it should be considered that this type of instrumentation is more complex and expensive than an electronic nose.

The aim of the present study was to develop a non-destructive method, based on MOS sensors, capable of classifying oils containing the typical virgin olive oil defects according to their sensory threshold previously established by trained panellists. For this purpose, linear discriminant analysis (LDA) models and artificial neural network (ANN) method were used. On the other hand, multiple linear regression (MLR) models were also constructed to predict the defect percentage added to sunflower oil.

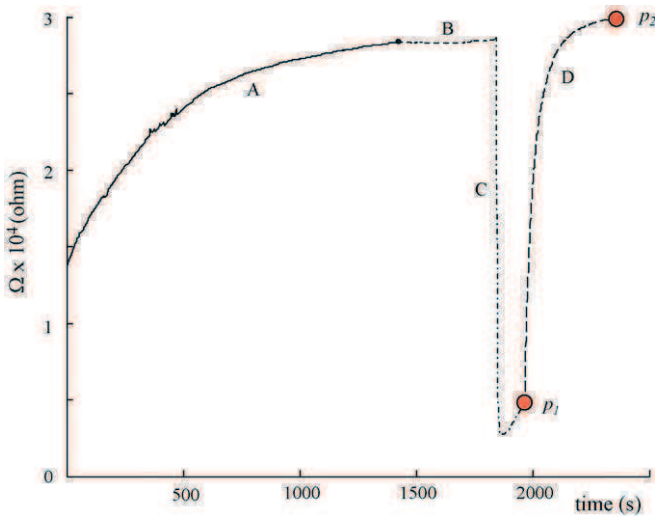

Fig. 1. Plot representing the electric resistance (Ω) of a MOS sensor during oil evaluation: (A) conditioning phase, (B) before injection phase, (C) measurement cycle and (D) recovery phase.

2. Materials and methods

2.1. Instrumentation and working conditions

An electronic olfactory system (EOS 507, Sacmi Imola S.C., Imola, Bologna, Italy) composed of a measuring chamber with 6 metal oxide sensors and a personal computer was used for the acquisition and analysis of the data generated by the EOS 507. The sensors used were: sensor 1 (SnO_2), sensor 2 ($SnO_2 + SiO_2$), sensor 3, 4 and 5 (catalyzed SnO_2 with Au, Ag and PD, respectively) and sensor 6 (WO_3). During the analysis, sensors were maintained at a temperature range of 350–450 °C. The EOS 507 was controlled by an integrated PDA equipped with proprietary software, and was connected to an automatic sampling apparatus (Model HT500H) which had a carousel of 10 sites for loading samples. Samples were kept at controlled temperature (37 °C) and placed in a chamber provided by a system that removes humidity from the surrounding environment.

2.2. MOS sensor array procedure

For each sample, 15 g were placed in 100 mL Pyrex vials equipped with a pierceable silicon/Teflon cap. For each sensor, signal is divided in four parts (see Fig. 1): (A) conditioning phase (25 min period employed to obtain a constant baseline), (B) before injection phase (in which samples were incubated at 37 °C for 7 min before injection), (C) measurement cycle (in which the oil headspace, sampled with an automatic syringe, was then pumped over the sensor surfaces for 2 min during which the sensor signals were recorded; in this phase, sensors were exposed to filtered air at a constant flow rate of 50 sccm (standard cubic cm per min) to obtain the baseline) and (D) recovery phase (another 7 min period applied to restore the original MOS conditions). Ambient air filtered with activated silica and charcoal was used as a reference gas during the recovery phase of the measurement cycle. The previous conditions ensured that the baseline reading had indeed been recovered before the next analysis was performed.

The experimental conditions adapted from Camurati et al. [10] were used, being each sample evaluated in triplicate in different days.

2.3. Determination of the sensory threshold by trained panelists

Sensory analysis was performed according to the European normative reported in Reg. 2568/91/EEC (Annex XII) [21]. Thresholds of the following defects (fusty, mouldy, muddy, rancid and winey) were assessed by ten trained assessors working as the professional panel of the Dipartimento di Scienze degli Alimenti (recognized by Italian Ministry-Mipaaf on 20 July 2006) according to the method described by Doc. 14, Rev. 2 (International Olive Council, 2007) [22]. The panel leader has used as reference defected oils those provided by IOC. A series of 12–15 samples of each of the defected oils were performed at descending concentrations by making successive dilutions in sunflower oil until the defect is not perceived. Then, paired comparison tests were carried out between these defected samples and a blank to establish the mean threshold of the panel. Up to a total of 8 pairs (the 8 samples with lower defect and 8 blanks) were randomly presented in successive independent tastings. After each tasting, the assessors were asked whether the two samples are identical or different. Upon completion of the test, the panel leader noted down the correct answers of the tasters for each concentration and expressed them as a percentage. The leader plotted the concentrations tested along the *x*-axis and the percentages of correct answers along the *y*-axis and then, by interpolation of the curve, shall determine the detection threshold which is the concentration corresponding to 75% correct answers.

2.4. Data treatment and statistical analysis

The data from the electronic nose was extracted and analyzed with the statistical package "Nose Pattern Editor" (Sacmi Imola S.C.). A feature extraction algorithm called "classical after feature" was applied to the data before other statistical treatments.

The response extracted by each sensor was defined by:

$$X = \frac{p_1}{p_2}$$

where p_1 was the resistance (see Fig. 1) of a sensor in the presence of the volatile compounds emitted from the VOO headspace, p_2 was the resistance of the sensor after the measurement (see Fig. 1), and X was the response of each sensor recorded.

In order to reduce the variability associated to possible fluctuations in the electronic nose signals, and to minimize other sources of variance also affecting the total signal of the sensors, normalized rather than absolute signals were used to construct LDA and MLR models. In order to normalize the variables, the signal of each sensor was divided by each one of the signals of the other 5 sensors; in this way, and taking into account that each pair of signals should be considered only once, $(6 \times 5)/2 = 15$ non-redundant signal ratios were obtained to be used as predictors.

LDA and MLR models were constructed using SPSS (v. 11.5, Statistical Package for the Social Sciences, Chicago, IL, USA). LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained [23]. This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of λ_w approaching zero are obtained with well resolved categories, whereas overlapped categories approach a λ_w of one. Up to N-1 discriminant vectors are constructed by LDA, and N has the lowest value for either the number of predictors or the number of categories. The selection of the predictors to be included in the LDA models was performed using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or *F*-test. However, the entrance of a new predictor modifies the significance of those predictors which are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. Probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted. On the other hand, to select the predictors used in the construction of MLR models, the SPSS stepwise algorithm with probability values, $F_{in} = 0.05$ and $F_{out} = 0.10$, was again used.

ANN models were constructed using STATISTICA Neural Networks 4.0 (Statsoft Inc.) ANN is a mathematical algorithm which has the capability of relating the input and output parameters, learning from examples through iteration without requiring a prior knowledge on the relationships between the process variables [24]. Studies have been reported for the classification of several agricultural products, such as fruit [25,26], fish and meat [27], and vegetables [28,29].

A MLP with Back-propagation learning algorithm was performed to set up networks capable of classifying samples with a defect and samples without defect (classified according to the panel sensory thresholds). The input values were represented by the response extracted by each sensor. Two nominal output variables (V_1 and V_2) were used to perform classification tasks: V_1 for the samples with a defect (category 1) and V_2 for the samples without defect (category 2).

The original dataset (category 1 and category 2 cases) was randomly divided into *training set* (60%), *verification set* (20%) and *test set* (20%). The *verification set* was used to identify the best network on the basis of the network's error performance and to stop *training* if over-learning occurs; the *test set* was performed to give an independent assessment of the network capability of classifying samples with a defect and samples without defect.

3. Results and discussion

3.1. Establishment of the sensory threshold by the sensory panel

The evaluation of olfactory sensory threshold of a panel is necessary to know the sensory capability of the assessors. In fact, this estimation represents the first step that drives the panel to receive the habilitation as official or professional panel (certified by a certification organism).

Fig. 2 shows the results of the evaluation of the sensory thresholds of the professional panel of the Dipartimento di Scienze degli Alimenti for the five VOO defects. In particular, the sensory thresholds of the panel (corresponding to the defect percentage perceived from at least 75% of the judges) followed the order: winey = fusty < mouldy < rancid < muddy. The thresholds were similar for winey (0.09%), fusty (0.09%), mouldy (0.12%) and rancid (0.13%) defects, whereas for muddy defect this value was higher (0.80%). As reported by Morales et al. [5] each defect is characterized by the contemporary presence in the head-space of the sample of several volatile compounds, but only some of these really contribute to the specific perception as a consequence of their individual concentrations and odour thresholds [5].

3.2. Classification of the oils containing the VOO defects according to their sensory threshold establish by the sensory panel

According to the panel taster group, the samples with a defect and a correct answer percentage higher than 75% were grouped as category 1 (samples that were over the sensory threshold), while the remaining samples were grouped as category 2. For example, for the rancid defect, samples prepared with 0.2, 0.4, 0.8, 3.12 and 6.25%

Table 1
Wilk's lambda (λ_w) values and predictors selected with their corresponding standardized coefficients of the LDA models constructed for each sensory defect.

	Fusty	Mouldy	Muddy	Rancid	Winey
	λ_w				
	0.166	0.167	0.029	0.041	0.184
Predictors[a]					
S1/S2	2.60	–	–	3.99	–
S1/S3	−3.63	7.47	5.76	13.91	2.48
S1/S4	−1.94	10.73	–	–	–
S1/S5	–	−6.62	−7.33	−17.22	–
S1/S6	4.55	−11.29	–	–	–
S2/S3	–	–	−9.82	6.29	–
S3/S5	–	–	–	–	2.96
S4/S5	–	–	11.01	–	–

[a] Ratios of sensor signals.

Table 2
Results of ANN: correct classified cases (%) for each sensory defect. training, verification and test.

	Correct classified cases (%)		
	Training	*Verification*	*Test*
Rancid	100	100	80
Mouldy	100	85	85
Muddy	100	80	80
Fusty	85	85	85
Winey	75	75	75

defect in sunflower oil corresponded to category 1, while those with 0, 0.04 and 0.09% corresponded to category 2. Then, using the normalized variables, LDA models capable of classifying the samples according to these categories were constructed. Five matrices were performed, one for each sensory defect. Each matrix contained 24 points (8 samples × 3 replicates) and 15 predictors. Another column containing the two categories previously described was added to each matrix.

The λ_w values, the variables selected by the SPSS stepwise algorithm and the corresponding model standardized coefficients, showing the predictors with large discriminant capabilities for each defect, are given in Table 1. As only two categories were used to construct the model, only one discriminant function can be obtained. As observed, λ_w values were in all cases lower than 0.2. As also observed, the ratio between sensors 1 and 3 (S1/S3) showed a high discriminant power for all the models. When leave-one-out validation was applied to each model, all the samples were correctly classified. As an example, a plot showing the LDA model constructed for the rancid defect is shown in Fig. 3. As observed, a good resolution was obtained.

The structure of the best MLP neural networks tested to classify samples with a defect and samples without defect is shown in Fig. 4. The structure is characterized by 6 neurons in the input layer and 8 neurons in the hidden layer. For any defect considered, the number of iterations needed to achieve the best result is 50. Results of *training*, *verification* and *test* of the best MLP neural networks are summarized in Table 2. These values were obtained with a momentum value of 0.3 and a learning rate of 0.1.

As showed in Table 2, the performed MLP neural networks appear to classify the samples with an acceptable performance. In particular, from *test* validations, about 100, 85, 85, 80, and 75% of the data set is correctly classified, respectively for rancid, mouldy, fusty, muddy and winey defect.

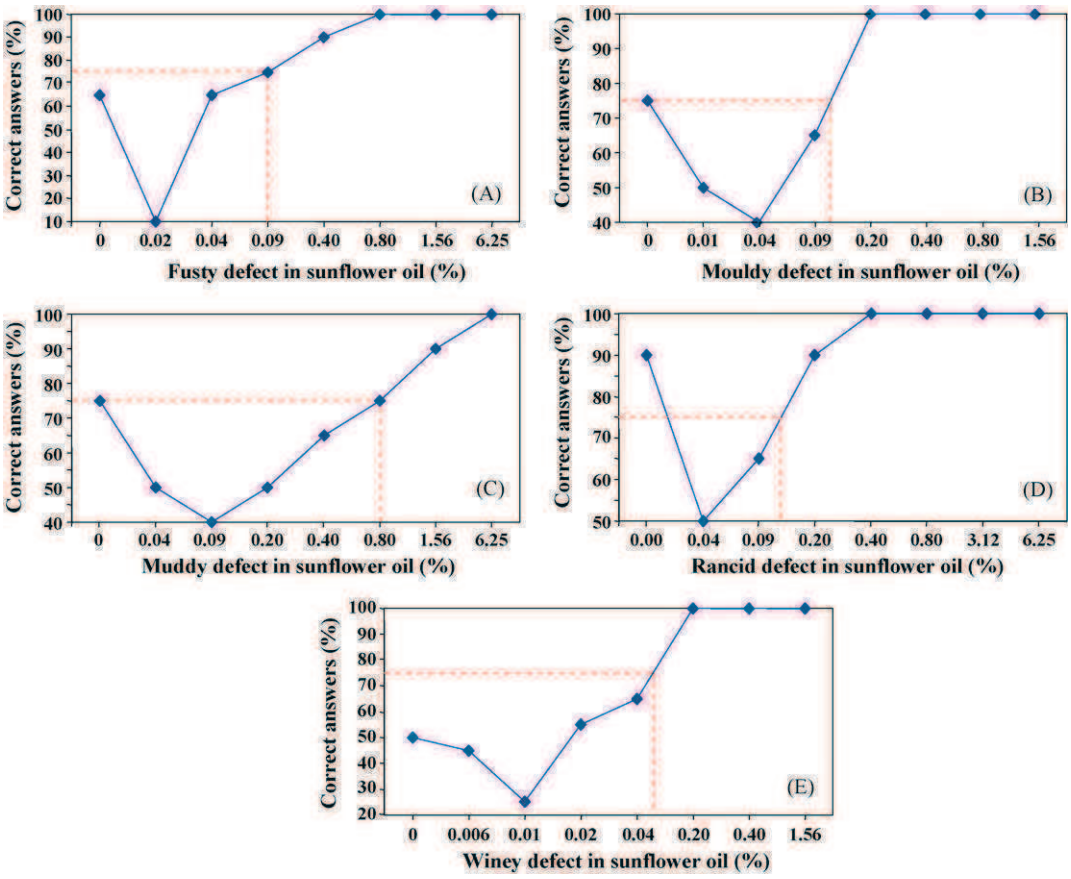

Fig. 2. Plots representing the defect (fusty, A; mouldy, B; muddy, C; rancid, D; winey, E) against the correct answer percentages, showing the detection threshold (defect % corresponding to 75% correct answers).

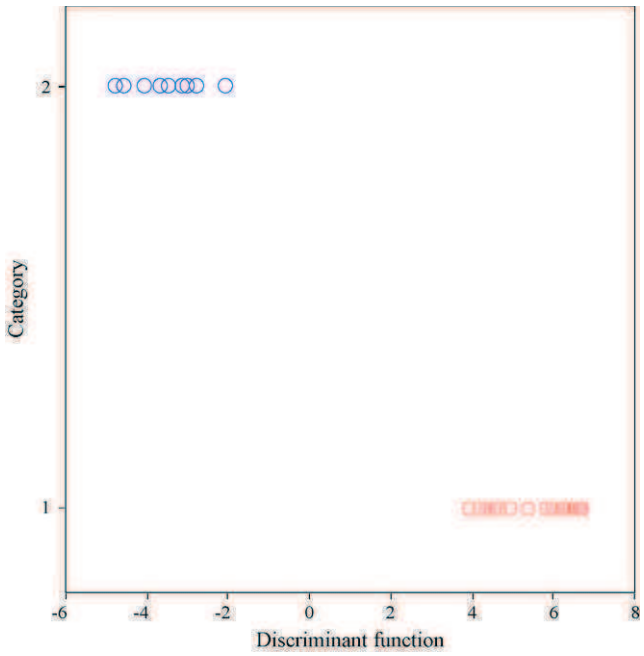

Fig. 3. Score plot on the plane of the discriminant function versus the category of the LDA model constructed to classify defected oil samples according to detection threshold of sensory analysis. Samples with a defect higher than detection threshold were grouped as category 1, the lower as category 2.

3.3. *Prediction of the defect percentage in sunflower oil by electronic nose data and MLR analysis*

The possibility of predicting the defect percentage added to the sunflower oil using electronic nose data was tried. For this purpose, the samples firstly described (8 samples for each defect) were used. Two additional samples were performed for each defect in order to reduce the weight of the samples with higher defect percentages. To construct MLR matrices, only the means of the replicates of the samples were included (10 objects for each model), which was important to reduce the number of variables selected by the SPSS stepwise algorithm during model construction. The 15 signal ratios described in Section 3.2 were also used as predictors to construct the MLR models.

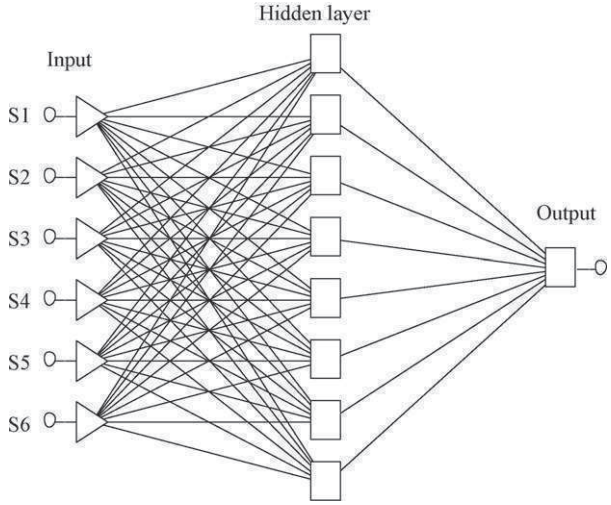

Fig. 4. MLP neural network structures used to classify defected oil samples.

The correlation plots of the calculated versus the experimental defect percentages are shown in Fig. 4. The regression coefficients (r), average prediction errors (*av. err.*, calculated as the sum of the absolute differences between expected and calculated defect percentages divided by the number of predictions) and the predictors selected for each MLR model with their corresponding non-standardized coefficients are detailed in Table 3. As observed, r values were in all cases higher than 0.988, being especially higher

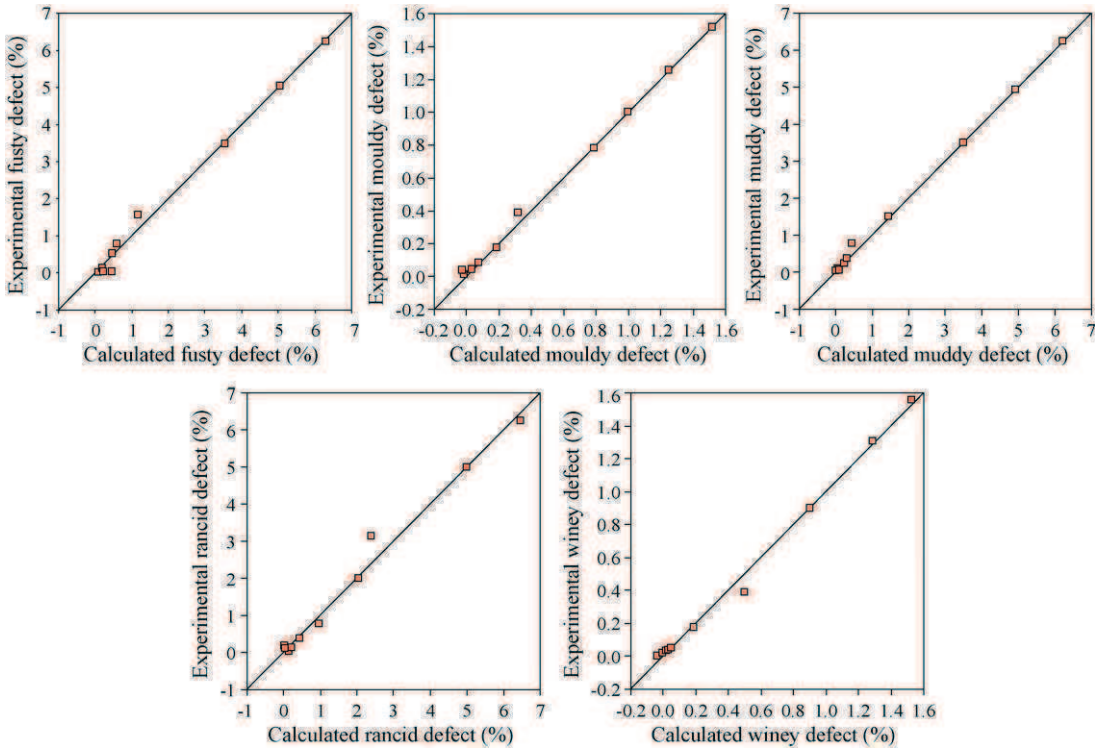

Fig. 5. Correlation plots of the calculated versus the experimental defect percentages.

Table 3
Regression coefficients (*r*), average prediction errors (av. err.) and predictors selected with their corresponding non-standardized coefficients for the MLR models constructed to predict defect percentage.

	Fusty	Mouldy	Muddy	Rancid	Winey
r	0.993	0.997	0.997	0.988	0.996
Av. err.(%)	0.51	0.22	0.43	0.90	0.39
Predictors[a]					
Constant	−651.19	14.74	−440.80	20.18	−66.64
S1/S4	–	–	–	−61.24	–
S3/S4	–	–	–	–	27.25
S3/S6	–	–	–	37.81	–
S4/S5	–	50.46	177.74	–	41.20
S4/S6	657.69	−138.47	–	–	–
S5/S6	–	75.27	271.62	–	–

[a] Ratios of sensor signals.

for fusty, mouldy, muddy and winey defects, with values higher than 0.993. This good correlation can be observed in Fig. 5. On the other hand, and as observed in Table 3, the regression models for the fusty defect (which was mainly characterized by the presence of some volatile compounds originated by fermentation processes, i.e. some branched C_5 components as 3-methyl butan-1-ol) and mouldy defect (produced by specific mould enzymes that produce volatile compounds such as 1-octen-3-one and 1-octen-3-ol) were mainly constructed with S4/S6 ratio (being S4 and S6 constructed with SnO_2 catalyzed with Au and WO_3, respectively), muddy defect (which was characterized by some volatile compounds originated by fermentation processes of oils stored for a long time on their sediment, i.e. propyl-propionate, ethyl-butanoate, propyl-butanoate and butyl-butanoate) by S5/S6 (SnO_2 catalyzed with Pd and WO_3, respectively), rancid defect (produced by several saturated and unsaturated aldehydes, such as nonanal and *E*-2-heptenal, respectively) by S1/S4 (SnO_2 and SnO_2 catalyzed with Ag, respectively) and winey defect (produced by acetic acid and ethyl acetate which were formed by sugar fermentation) by S4/S5 ratio (SnO_2 catalyzed with Ag and Pd, respectively).

4. Conclusions

This work demonstrates the ability of the electronic nose to evaluate the presence of the most common defects of VOO (fusty, mouldy, muddy, rancid and winey) when tested in different concentrations. The sensory analysis performed by a panel of trained assessors could be made more robust by the jointly use of an electronic nose, which was able to test singular defects one-by-one and to classify the defected oils according to their sensory threshold. On the other hand, excellent predictions of the defect percentage were obtained using MLR models. Then, the electronic nose, if correctly trained, could be considered an useful tool to work in parallel to panellists, for example to realize a rapid screening of large set of samples with the aim to discriminate defective oils. Under this point of view it could be helpful to test the panel performance.

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Biographies

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T. Gallina Toschi received bachelor degree in pharmaceutical chemistry on 1990. From 1990 to 1993 she studied for a post-doctoral degree in food technology of the University of Bologna and she worked on cholesterol and fatty acids oxidation. She is an assistant professor in the Department of Food Science at the University of Bologna since 1995. Her research activity is focused on: lipid analysis of vegetable oils; composition of food lipids, modification and degradation of components caused by processing and preservation. She worked using various absolute (FT-IR, FT-NIR), preparative (TLC, SPE) simple and hyphenated (GC, GC–MS, HPLC-UV–VIS, HPLC-ELSD, HPLC-DAD/FD, HPLC–MS-ES, HPLC–MS-APCI and LC–GC) analytical techniques.

ARTICOLO 4

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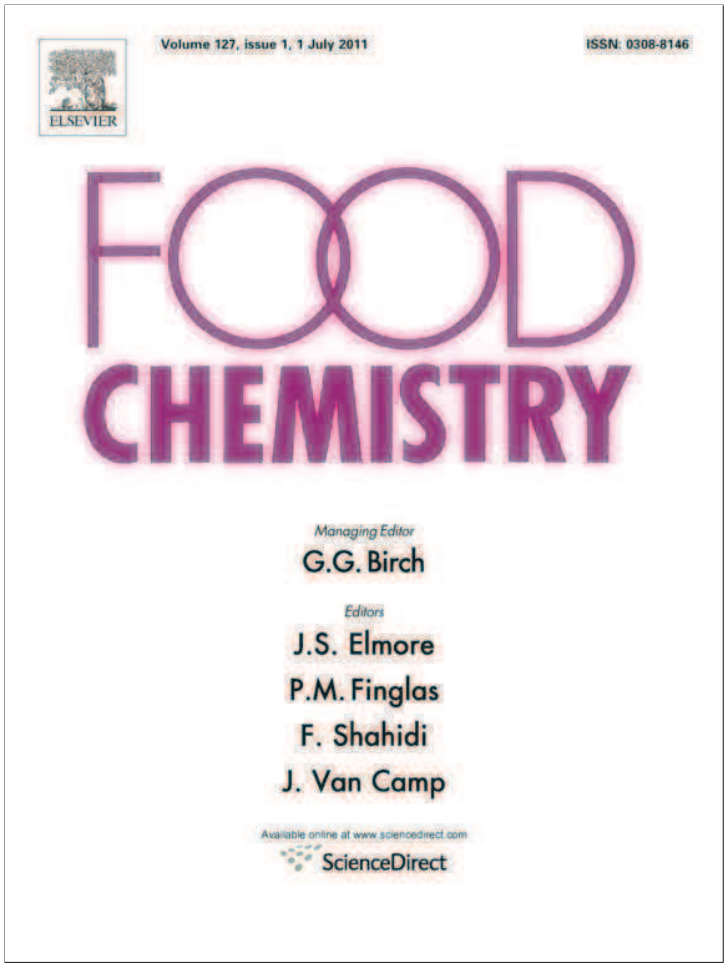

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Analytical Methods

A spectroscopic and chemometric study of virgin olive oils subjected to thermal stress

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ABSTRACT

The paper describes a study of thermal stress of three different samples of virgin olive oil in terms of oxidative stability. Fatty acid composition, evaluation of oxidative stability under forced conditions (OSI), determination of UV-spectrophotometric oxidation indexes (k_{232} and k_{270}) and spectral properties were explored along the thermal treatment. The samples were subjected to heating treatment at 180 °C and evaluated after 0, 30, 60, 90, 120, 150 and 180 min. Middle infrared (MIR) and visible–near infrared (Vis–NIR) spectra were elaborated by partial least squares modelling to individualise regions and bands where critical variations were present. Two bands were found as principal influential ones (1245–1180 cm^{-1} and 1150–1030 cm^{-1}) on MIR while one primary region was identified on Vis–NIR (2200–1325 cm^{-1}).

1. Introduction

Extra virgin olive oil (EVOO) is widely known because of its high health benefits and sensory quality in comparison to other oils and fats (Bendini et al., 2007), especially due to the presence of a high ratio between monounsaturated and polyunsaturated fatty acids and its antioxidant fraction (principally lipophilic and hydrophilic phenolic compounds) (Aparicio, Roda, Albi, & Gutiérrez, 1999).

Virgin olive oil is a principal ingredient in the Mediterranean diet and it is consumed in different ways: raw in salads, on traditional food (i.e. as breakfast in "tostada" in the South of Spain or as meal in pasta or "bruschetta" in the South of Italy), toasts and other foodstuffs (Cerretani, Biasini, Bonoli-Carbognin, & Bendini, 2007), but often it is also consumed after domestic heating, such as fried, boiled or after conventional and microwave heating (Brenes, García, Dobarganes, Velasco, & Romero, 2002). These thermal treatments are commonly utilised for home-cooking, food catering and industrial processes (Brenes et al., 2002; Carrasco-Pancorbo et al., 2007; Cheikhousman et al., 2005). Several studies published in literature up to now have compared the effects of conventional and microwave heating on the physical and chemical parameters of extra virgin olive oil (Albi, Lanzón, Guinda, Pérez-Camino, & León, 1997; Brenes et al., 2002; Caponio & Gomes, 2001; Vieira & Regitano-D'Arce, 1999; Valli et al., 2010).

It has been observed that heating can affect the phenolic fraction and the oxidation stability and degradability of oil. In fact, Cerretani, Bendini, Rodriguez-Estrada, Vittadini, and Chiavaro (2009) have studied the effect of microwave heating treatments on phenols compared to the effects produce by oxidation or heating by conventional oven; particularly lignans have shown the highest stability to the thermal treatments due to their strong antioxidant properties (Carrasco-Pancorbo et al., 2007). The parameters that have been proven to influence the extent of oxidation and the degradation of oils in a highest extension during heating are oil composition, time and temperature of heating, food (in the case that some food is in contact with the oil), ratio between surface and volume of the oil (Andrikopoulos, Kalogeropoulos, Falirea, & Barbagianni, 2002; Christie, Brechany, Sebedio, & Le Quere, 1993). However, strong interactions exist among these variables and because of that, they are difficult to control and define (Jorge, Márquez-Ruiz, Martín-Polvillo, Ruiz-Méndez, & Dobarganes, 1996).

More recently, Valli et al. (2010) have studied the phenolic fraction of two EVOO and their admixtures after thermal treatments using microwave and conventional oven by HPLC-DAD/MSD and NMR spectroscopy. In this work authors have observed an increase of the dialdehydic forms of secoiridoids (dialdehydic form of elenolic acid lacking a carboxymethyl group, EDA; *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid,

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p-HPEA-EDA) after microwave and conventional heat treatments. It is likely that reasonable chemical conversions from elenolic acid (EA), *p*-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA), and 3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA) to their respective dialdehydic forms (EDA; *p*-HPEA-EDA; 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid, 3,4-DHPEA-EDA) were induced by heating.

Other analytical techniques as FT-IR have been recently applied to the analysis of phenolic fraction of EVOO (Cerretani et al., 2010). Spectroscopic FT-IR coupled with chemometrics methods have been successfully used to detect olive oil adulteration (Lerma-García, Ramis-Ramos, Herrero-Martínez, & Simó-Alfonso, 2009; Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010; Ozen & Mauer, 2002) and freshness (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007). The chemometric algorithm partial least square (PLS) has been repeatedly and extensively used to obtain different quality parameters of edible oils (Al-Alawi, Van de Voort, & Sedman, 2004; Iñón, Garrigues, Garrigues, Molina, & De la Guardia, 2003; Li, Van de Voort, Ismail, & Cox, 2000b; Li et al., 2000a).

In this work three different EVOOs have been subjected to heating treatment by conventional oven with the following aims:

(1) to study the changes in the oxidative stability (k_{232}, k_{270} and OSI time) and in the spectroscopic properties (Vis–NIR and FT-MIR) occurring in this process;

(2) to find, by spectroscopic analysis, the compounds or the families of compounds that are most important in this oxidation pathway;

(3) to explore the opportunity of adopting some rapid spectroscopic methods (Vis–NIR and FT-MIR) for the control of cooked olive oils.

2. Materials and methods

2.1. Apparatus

Fatty acid (FA) analyses were performed using an Autosystem XL Perkin Elmer (Shelton, CT, USA) gas chromatograph equipped with a flame ionisation detector (FID). The determination of k_{232} and k_{270} was carried out using an UV–vis 1800 instrument (Shimadzu Co., Kyoto, Japan), which had a six slot shuttle and a system for temperature control of working conditions. The oxidative stability of samples was evaluated using an eight-channel oxidative stability instrument (Omnion, Decatur, IL, USA). The FT-MIR spectra were acquired on a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid™ interferometer and a DigiTect™ detector system coupled to an attenuated total reflectance (ATR) accessory. NIR analysis was carried out by NIR Lab Near Infra Red by transmittance instrument (SACMI Imola S.C., Imola, Bologna, Italy).

2.2. Materials, reagents and standards

Potassium hydroxide, methanol, *n*-hexane, isooctane and acetone were purchased from Merck (Darmstadt, Germany). The standard mixture of FA methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA).

2.3. Samples and thermal treatment

Three different samples of extra virgin olive oil (named A, B and C) from different Italian regions (Abruzzo, Marche and Puglia) harvested in the fall of 2009, were analysed in this experimental study. The oils were different in terms of cultivar and ripening degree.

For analytical purposes, six aliquots (50 g) of each sample were inserted in 250 mL opened glass beakers (7.2 cm i.d.) and subjected to conventional heating at 180 °C in a oven (model M20-VN, Instruments s.r.l, Bernareggio, Milan, Italy). The beakers were removed from the oven at fixed intervals of 30 min, obtaining samples with different heating treatments (30, 60, 90, 120, 150, 180 min) to be analysed. All heated samples were cooled at room temperature (23 ± 1 °C) for 30 min and stored in bottles without headspace at 12 °C before chemical analysis.

2.4. Fatty acid composition

The FA composition of oil samples was determined as fatty acid methyl esters (FAMEs) after alkaline treatment, obtained by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol, and subsequent gas chromatographic analysis, according to Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, and Lercker (2006), with slight modifications. Analytes were separated on a RTX-2330 capillary column (30 m × 0.25 mm i.d., 0.2 μm film thickness) from Restek (Bellefonte, PA, USA). Column temperature was held at 140 °C for 5 min and then it was increased at 2.5 °C min^{-1} until 240 °C. The FID and the injector temperatures were both set at 250 °C. Peak identification was accomplished by comparing the peak retention times with those of the GLC 463 FAME standard mixture, injected under the same gas chromatographic conditions. The GC response factor of each FA was also calculated by using the GLC 463 FAME standard mixture.

2.5. Determination of k_{232} and k_{270}

The UV-spectrophotometric indexes (k_{232} and k_{270}) were determined according to the European Communities official methods and the following amendments (European Union Commission, 1991). To calculate the k_{270} and k_{232} values, the oil samples were diluted in isooctane (1:100 v/v for k_{270} and 1:1000 v/v for k_{232}), placed into a 1 cm quartz cuvette, and analysed at the wavelengths of 270 and 232 nm, against a blank of isooctane. Three replicates were prepared and analysed per sample.

2.6. Evaluation of oxidative stability under forced conditions

An eight-channel oxidative stability instrument (OSI) (Omnion) was used. To obtain the OSI induction time, a stream of purified air

Table 1
FA composition, OSI values, k_{232} and k_{270} values of samples before the thermal treatment.[a]

	A	B	C
C16:0	15.43 ± 0.01	10.52 ± 0.02	12.79 ± 0.12
C16:1 A	0.13 ± 0.00	0.11 ± 0.00	0.12 ± 0.01
C16:1 B	1.27 ± 0.00	0.58 ± 0.00	1.07 ± 0.01
C17:0	0.06 ± 0.00	0.16 ± 0.00	0.04 ± 0.01
C17:1	0.10 ± 0.00	0.23 ± 0.00	0.08 ± 0.00
C18:0	2.01 ± 0.01	2.73 ± 0.02	1.78 ± 0.01
C18:1 *n*−9	65.64 ± 0.01	76.01 ± 0.07	72.81 ± 0.13
C18:1 *n*−7	3.26 ± 0.01	1.68 ± 0.03	2.79 ± 0.02
C18:2	10.34 ± 0.01	6.40 ± 0.01	7.10 ± 0.03
C20:0	0.38 ± 0.00	0.40 ± 0.00	0.30 ± 0.00
C18:3 *n*−3	0.95 ± 0.00	0.77 ± 0.00	0.76 ± 0.01
C20:1	0.29 ± 0.01	0.27 ± 0.00	0.26 ± 0.01
C22:0	0.12 ± 0.00	0.13 ± 0.00	0.09 ± 0.00
OSI time (h)	11.35	32.15	20.20
k_{232}	2.59 ± 0.10	2.29 ± 0.35	3.23 ± 0.40
k_{270}	0.10 ± 0.01	0.15 ± 0.01	0.26 ± 0.00

[a] Data are expressed as mean of three determinations, with the standard deviations (except for OSI time). Fatty acid composition, determined by gas chromatography analysis and expressed as percentages; OSI time, oxidative stability index expressed as hours and hundredths of hours; k_{232} and k_{270} values determined by spectroscopic analysis.

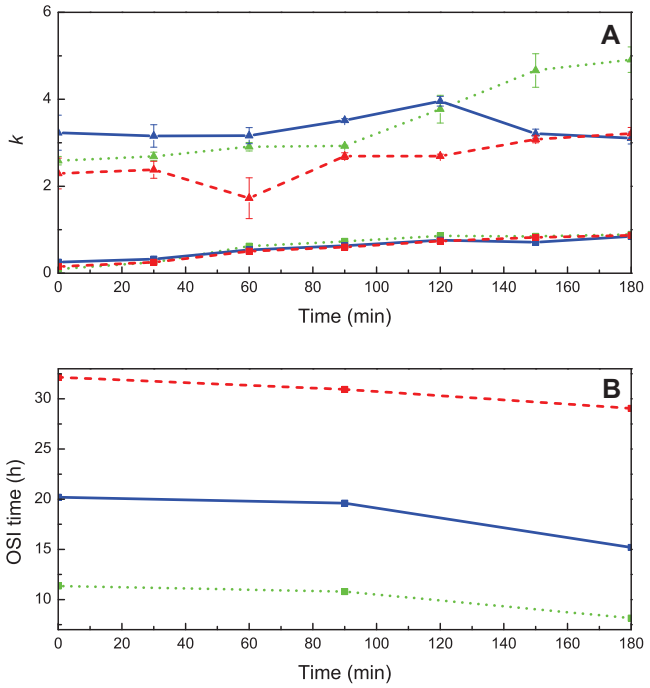

Fig. 1. (A) Plot of k_{232} (▲) and k_{270} (■) measured at heating times 0, 30, 60, 90, 120, 150 and 180 min for samples A (···), B (– –) and C (—). (B) OSI time values (h), of samples A (···), B (– –) and C (—) monitored at heating times 0, 90 and 180 min.

(120 mL min^{-1} air flow rate) was passed through 5.0 ± 0.1 g oil sample heated at 110 ± 0.1 °C, under atmospheric pressure. The effluent air contains especially short chain acids as formic acid and other volatile compounds formed during thermal oxidation of the oil; these substances were recovered and measured in deionised water, as an increase of conductivity. The OSI time was expressed in hours and hundredths of hours, which was defined as a measure of the oxidative stability of oil.

2.7. FT-MIR spectroscopy

A small amount of the oil samples (about 1 g) was uniformly deposited on the crystal surface of ATR accessory (Specac Inc., Woodstock, GA, USA), equipped with a ZnSe 11 reflection crystal. Analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the wavenumber range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, and the data exported by OPUS r. 6.0 (Bruker Optics) software in ASCII compatible format. For each sample, the absorbance spectrum was collected against a background, obtained with a dry and empty ATR cell. Three spectra per sample were recorded. After acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

2.8. Vis–NIR spectroscopy

The samples were inserted in cuvettes (optical glass; light path: 20 mm; Hellma, Jena, Germany). Spectra were acquired using the halogen lamps as light source and an optical filter of 200 FN, a measuring time of 6 ms, within a wavenumber range of 33,000–9000 cm^{-1}. Three spectra per sample were recorded in ASCII compatible format. After acquiring each spectrum, the glass container was cleaned with *n*-hexane, soap and then rinsed with acetone.

2.9. Chemometrics methods

PLS regression aims to find the relationship between a set of predictor (independent) data, X $(m \times n)$, and a set of responses (dependent), Y $(m \times l)$. Here, n and l are the independent and dependent variables, respectively, and m is the observation vector.

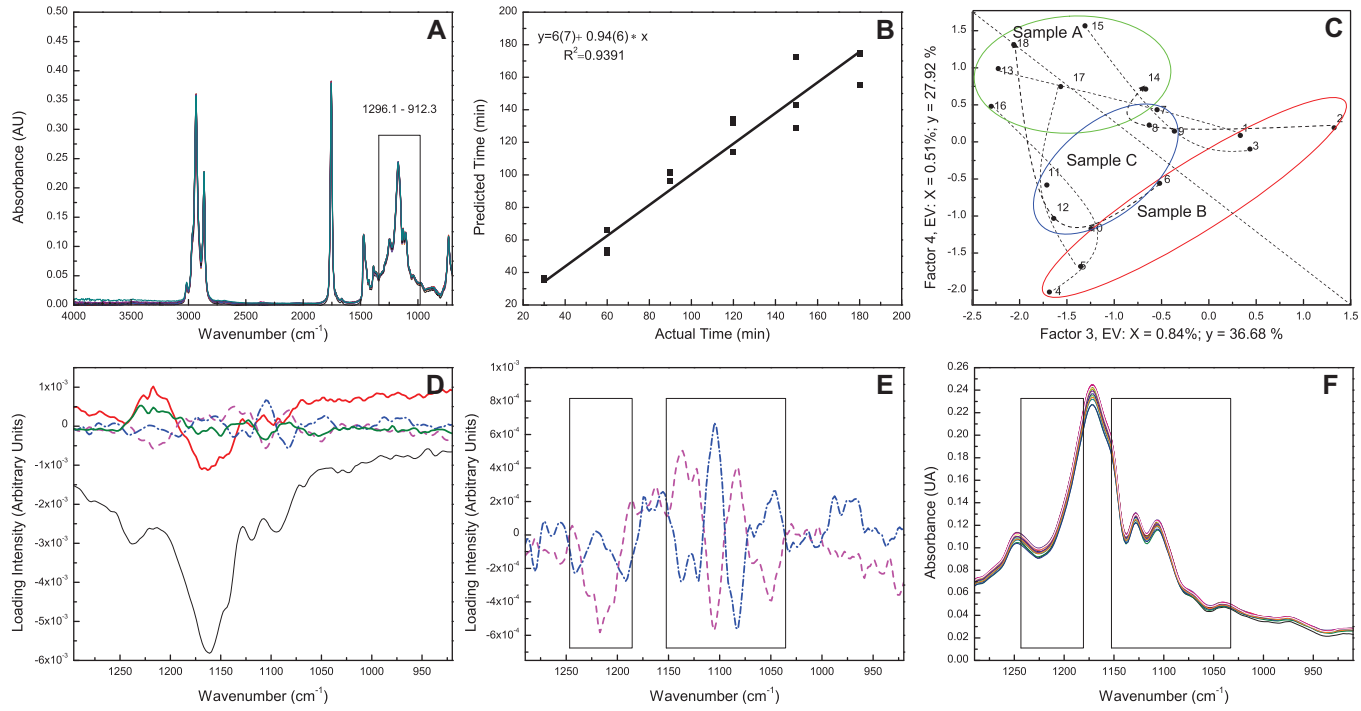

Fig. 2. (A) Full FT-MIR spectra (4000–700 cm^{-1}). (B) Actual vs. predicted heating time values in the calibration set in the selected spectral range (1296.1–912.3 cm^{-1}). (C) Third and 4th PLS factor score plot, see reference number in Table 2. (D) First 5 PLS loadings vectors. (E) Third and 4th PLS loadings vectors. (F) FT-MIR spectra in the selected wavenumber range (1296.1–912.3 cm^{-1}).

Table 2
Code Numbers used for each sample in PLS score plot of MIR and PLS score plot of NIR.

Code	PLS-FT-MIR	Time (min)	Code	PLS-NIR	Time (min)
1	B	120	1	B	120
2	B	150	2	B	150
3	B	180	3	B	180
4	B	30	4	B	30
5	B	60	5	B	60
6	B	90	6	B	90
7	C	120	7	C	120
8	C	150	–	–	–
9	C	180	8	C	180
10	C	30	9	C	30
11	C	60	10	C	60
12	C	90	11	C	90
13	A	120	12	A	120
14	A	150	13	A	150
15	A	180	14	A	180
16	A	30	15	A	30
17	A	60	16	A	60
18	A	90	17	A	90

However, it differs from the multiple linear regression technique (MLR) mainly that PLS is able to give stable predictions even when *X* contains highly correlated variables. Detailed description of PLS method and its algorithms could be found elsewhere (Haaland & Thomas, 1988).

The chemometrics computations were performed in Matlab 7.0 (Mathworks, Inc., Natwick, MA, USA). Variable selection was implemented using the graphical interface MVC1 provided by Olivieri, Goicoechea, and Iñón (2004). A PLS Score Plots was performed using Tomcat Toobox (Daszykowski et al., 2007). PLS was run on mean-centred data.

All programs were run on an ACER-Aspire 5050 computer with an AMD Turion™ 64 Mobile, 2.20 GHz microprocessor and 2.00 Gb of RAM.

3. Results and discussion

3.1. Compositional analysis

Table 1 groups the fatty acid composition, the OSI time, and the k_{232} and k_{270} coefficients for the three olive oils studied. Before thermal stress only sample B showed k_{232} and k_{270} values that were both below the limits established by the EC Regulation for EVOO category (European Union Commission, 2007), which corresponded to 2.50 and 0.22, respectively. Sample C showed both values over these limits, while sample A exceeded the limit only for k_{232} (Table 1).

Oxidative status of the samples were evaluated by monitoring the trend of conjugated dienes (k_{232}) and trienes (k_{270}) during the thermal stress (Hrncirik & Fritsche, 2005; Lerma-García, Simó-Alfonso, et al., 2009; Mancebo-Campos, Fregapane, & Desamparados Salvador, 2008) and analysing the samples heated at different times of treatment (Fig. 1, part A). The three samples underwent a significant increase of k_{270} with heating (Allouche, Jiménez, Gaforio, Uceda, & Beltràn, 2007; Bendini, Valli, Cerretani, Chiavaro, & Lercker, 2009), reaching values higher than the legal limit after only 30 min of thermal treatment (see Fig. 1, part A). During heating, a common trend for k_{232} among samples was not evidenced (Fig. 1, part A). As reported in Fig. 1, part B, the OSI time (h) was also checked halfway through the treatment (90 min) and at the end (180 min). Before the thermal treatment, the three samples showed high differences among OSI values: such a difference in oxidative stability could be easily related to the FA composition of the oils (Table 1), as previously reported in literature (Aparicio et al., 1999; Lerma-García, Simó-Alfonso, et al., 2009). In fact, sample B showed the highest OSI time value (32.15 h), the highest oleic acid content and the lowest amounts of polyunsaturated FA (linoleic and linolenic acids). Moreover, at the end of the thermal treatment sample B was the one that registered the lowest decrease (9.64%) in terms of OSI time, confirming its remarkable oxidative

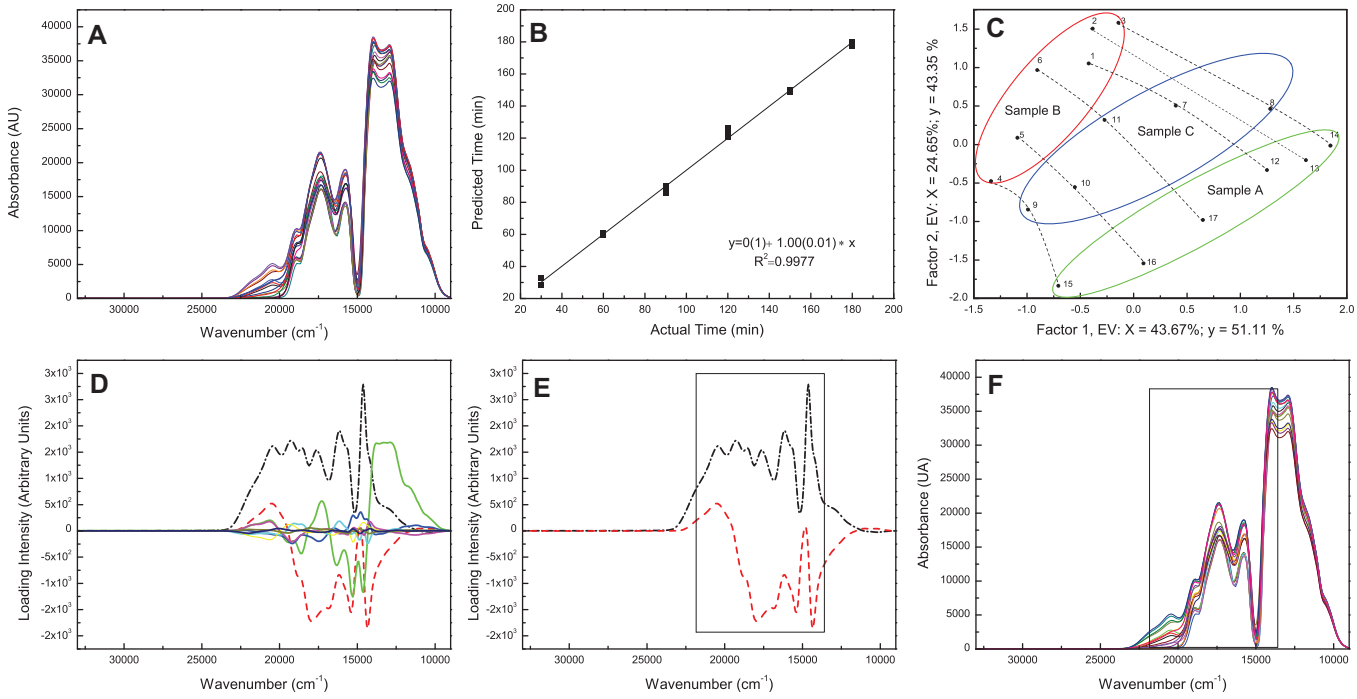

Fig. 3. (A) NIR spectra (33,000–9000 cm^{-1}). (B) Actual vs. predicted heating time values in the calibration set. (C) First and 2nd PLS factor score plot, see reference number in Table 2. (D) First 9 PLS loadings vectors. (E) First and 2nd PLS loadings vectors. (F) NIR spectra in the selected wavenumber range.

stability, while sample A and sample C presented a OSI time decrease of 28.19% and 24.75%, respectively (Fig. 1, part B).

3.2. Spectral analysis

The analysis of the variations in the FT-MIR spectra is not very easy, because these changes are very weak and there are so many peaks and shoulders without resolution (Fig. 2, part A). For a better visualisation of trends, the compression of the spectral information is needed. Principal component analysis (PCA) is one of the most used tools for this kind of works. Although, in this particular case it has not been possible to find any correlation between the PCA scores and the heating time.

Therefore, PLS was used, because it is a tool capable of addressing decomposition of the spectra to the dependent variable (heating time) (Carlson & Gautun, 2005). Since PLS was unable to find any acceptable correlation between oil spectra evolution and heating time using full spectrum FT-MIR, we used a wavelength selection algorithm (Xu & Schechter, 1996). The region 1296.1–912.3 cm^{-1} was selected as the optimum for regression (Fig. 2, parts A and B). Then we proceeded to analyse the score plots of significant PLS factors to see which of them was correlated with both the original composition of the oil and the evolution in time (Fig. 2, part C and Table 2). A segmentation was pointed out to 3rd and 4th PLS factor (Fig. 2, part C), which can distinguish among the different oils A, B and C. Moreover, the first (0–90 min) and the last (120–180 min) heating times into each kind of oil could be distinguish, as is showed with a dashed line. Once these factors were individualised as the most relevant ones, their loadings were evaluated (Fig. 2, parts D and E). Two regions were identified as principal influential ones: 1245–1180 cm^{-1} and 1150–1030 cm^{-1}, as shown in Fig. 2, part F. These regions comprise the bands belonging to $-CH_2-$ (1238), C–O (1238, 1138, 1118, 1097 and 1033 cm^{-1}), being C–O reported by Lerma-García, Simó-Alfonso, Bendini, and Cerretani (2011), as one of the regions more affected by oxidation.

In the case of the NIR spectra, shown in Fig. 3, part A, there were not many peaks but the analysis of the spectral variations was also complicated due to the low resolution among them.

Therefore, for better visualisation of trends, PLS and PCA were applied. UV–NIR spectra did not show any correlation between PCA scores and the heating times, but PLS was able to find an acceptable correlation (see Fig. 3, part B) with full spectrum NIR. Because of that, we proceeded to analyse score plots of significant PLS factors (Fig 3, part C and Table 2) to see which of them were correlated with both the original composition of the oils A, B and C and the evolution in time. A segmentation was evidenced to 1st and 2nd PLS factor, which distinguished among the different oils. In addition, the different heating times appear sorted in ascending way with the 2nd PLS-factor for A, B and C oils (Fig. 3, part C and reference number in Table 2). This fact shows that the 2nd PLS-factor is very important for the correlation. Once these PLS factors were detected as the most relevant, their loadings were evaluated (Fig 3, parts D and E). The major influence region was individualised in: 2200–1325 cm^{-1} (Fig. 3, part F).

4. Conclusions

This work describes a study of thermal stress on three virgin olive oils. Evaluation of oxidative stability under forced conditions, determination of k_{232} and k_{270} and spectral properties were explored along the thermal treatment. Fatty acid composition was explored at the beginning of the work. The three samples underwent a significant increase of k_{270} with heating, while a common trend for k_{232} among samples was not evidenced. Before the thermal treatment, the three samples showed high differences among OSI values. At the end of the thermal treatment sample B, with highest initial OSI time, highest oleic acid and lowest amounts of polyunsaturated FA content, registered the lowest decrease (9.64%) in terms of OSI time. The opportunity to adopt rapid spectroscopic methods (Vis–NIR and FT-MIR) in the control of cooked olive oils has been explored by using the chemometric analysis PLS. The most significant regions included the bands related to $-CH_2-$ (1238) and C–O (1238, 1138, 1118, 1097 and 1033 cm^{-1}) groups, concluding that C–O was one of bands more affected by oxidation. The spectroscopic data elaboration by PLS was able to distinguish among the different oils and the different heating times. This approach could be useful for monitoring the oxidative status of cooked oils, both in industrial scale or in food-catering field.

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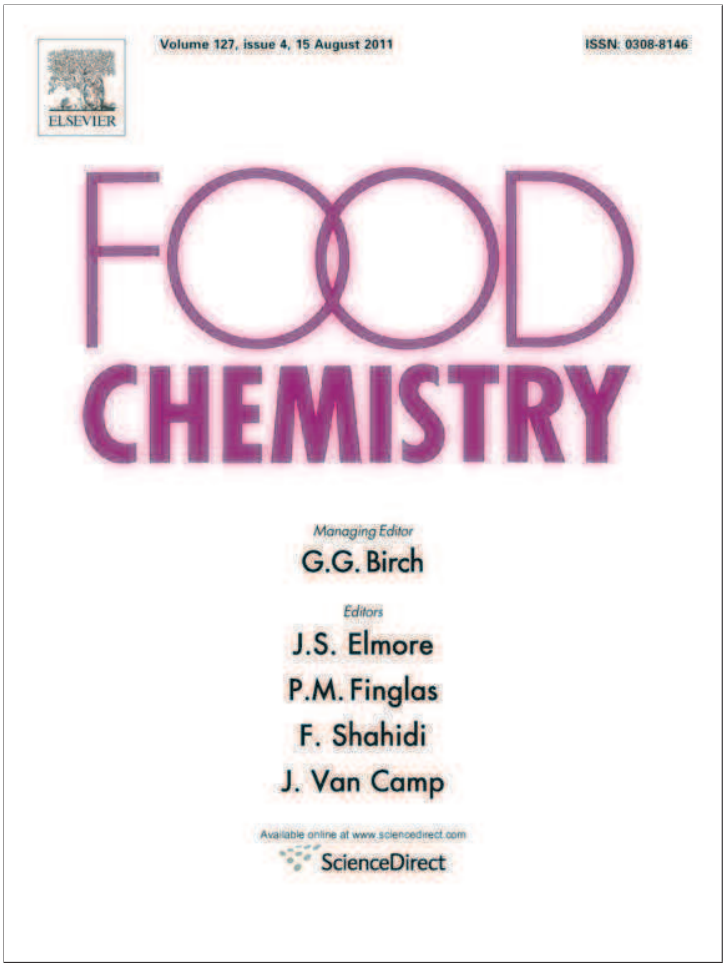
ARTICOLO 5

Cerretani, L., Maggio, R.M., Barnaba, C., Toschi, T.G., Chiavaro, E. Application of partial least square regression to differential scanning calorimetry data for fatty acid quantitation in olive oil. *Food Chemistry* 127 (4), pp. 1899-1904, 2011 (IF 3,655).



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Analytical Methods

Application of partial least square regression to differential scanning calorimetry data for fatty acid quantitation in olive oil

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ABSTRACT

A chemometric approach based on partial least (PLS) square methodology was applied to unfolded differential scanning calorimetry data obtained by 63 samples of different vegetable oils (58 extra virgin olive oils, one olive and one pomace olive oil, three seed oils) to evaluate fatty acid composition (palmitic, stearic, oleic and linoleic acids, saturated (SFA), mono (MUFA) and polysaturated (PUFA) percentages, oleic/linoleic and unsaturated/saturated ratios).

All calibration models exhibited satisfactory figures of merit. Palmitic and oleic acids, as well as SFA showed very good correlation coefficients and low root mean square error values in both calibration and validation sets. Satisfactory results were also obtained for MUFA, PUFA, stearic and linoleic acids, O/L ratio in terms of percentage recoveries and relative standard deviations. No systematic and bias errors were detected in the prediction of validation samples.

This novel approach could provide statistically similar results to those given by traditional official procedures, with the advantages of a very rapid and environmentally friendly methodology.

1. Introduction

Olive oil is the primary source of fat in the Mediterranean diet. It is consume has been associated with a reduced risk of overall and cardiovascular mortality, cancer frequency and incidence of Parkinson and Alzheimer disease, leading to an improved life quality (Covas, Konstantinidou, & Fitó, 2009). These health benefits are largely related to the high content of monosaturated fatty acids and oleic acid in particular, although it is now largely recognised the additional effect of other minor components having biological properties (Bendini et al., 2007). In addition, both the high content of oleic acid and low amounts of linoleic and linolenic acids, give an important contribution to the high oxidative stability to this type of vegetable oil (Aparicio, Roda, Albi, & Gutiérrez 1999).

Fatty acid composition of different commercial categories of olive oil, legally defined by the European Community (EC) Council of Regulation (EC, 2007) and also reported on the recently amended Current Official Standard of Codex Alimentarius (2009), is well recognised to largely vary. In particular, content of oleic acid ranges from 55% to 83%, as it is influenced by many agronomical factors (olive variety, climatic conditions during growth, degree of maturation) and practices related to irrigation treatment (Baccouri et al., 2007).

Differential scanning calorimetry (DSC) is widely employed in the field of vegetable oils (Aboul-Gheit, Abd-el-Moghny, & Al-Eseimi, 1997). Thermal properties obtained by cooling and heating thermograms are well recognised to be related to chemical composition for different vegetable oils (Che Man & Tan, 2002; Tan & Che Man, 2002) and a mathematical model based on a simple regression procedure was also developed to correlate melting parameters of several oils to mass fractions of mono and polyunsaturated fatty acids (Fasina, Craig-Schmidt, Colley, & Hallman, 2008). Relations have been also recently studied and established for extra virgin olive oil (Chiavaro et al., 2007) and commercial categories of olive oil (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008) as well as, more deeply, statistical correlations were found among thermal properties upon cooling and both major and minor components of this vegetable oil (Chiavaro, Rodriguez-Estrada, Bendini, & Cerretani, 2010).

DSC is a very well established technique with several advantages for its application, as it does not require chemical treatments or time-consuming manipulation practices before each measurement leading to an easy data collection. Otherwise, the chemometric processing of digitised DSC curves is not commonly employed in literature although this approach seems to be an attractive alternative to the classical chemical methods due to the

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advantages obtained by DSC application above discussed. Some examples are reported in the fields of pharmaceuticals and chemicals (Miltyk, Antonowicz, & Komsta, 2010; Smidt & Tintner, 2007) and a lower number on food matrices such as meat (Bertram, Wu, van den Berg, & Andersen, 2006; Nedenskov Jensen & Jørgensen, 2003).

On the other hand, the coupling of quantitative chemometrics strategies, partial least square (PLS) in particular, to evaluate the quality of edible oils by physical methods, such as spectrometric ones, are extensively reported in literature (Al-Alawi, Van de Voort & Sedman, 2004; Iñón, Garrigues, Garrigues, Molina, & de la Guardia, 2003). This combination allowed to merge spectral and analytical information leading to the construction of very useful predictive models for properties of interest generally based on concentration values. In particular, Maggio et al. (2009) has been recently applied coupled FTIR-PLS procedures to evaluate fatty acid composition and other quality parameters of virgin olive oil and to discover the adulteration of extra virgin olive oil added with other edible oils (Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010).

The DSC–PLS approach here proposed to quantify fatty acids represents an easy and convenient means for monitoring olive oil and, to the authors best knowledge, no works are present in literature on this application. Thus, the aim of this work was to develop and validate an analytical method based on DSC data, in combination with multivariate calibration methodologies, for the evaluation of important features of the olive oil's fatty acids composition. Palmitic, stearic, oleic and linoleic acids as well as total saturated, monosaturated and polyunsaturated percentages and some important ratios commonly employed to define olive oil quality were considered (oleic/linoleic acid and unsaturated/saturated).

2. Material and methods

2.1. Sampling

A series of 189 (63 by triplicate) samples of vegetable oils were analysed. Fifty-eight of them were extra virgin olive oils and differed in terms of cultivar, ripening degree, area of growth and extraction system (type, productive capacity and manufacturer), one was olive oil, one was obtained from pomace, while three were from other botanical origins (hazelnut, high oleic sunflower, canola oils). All Italian and Spanish extra virgin olive oils were obtained from olives hand-picked in the season 2008–2009 whereas Tunisian extra virgin olive oils, pomace olive oil, olive and all seed oils, were obtained from crop season 2007–2008. Samples were all stored in dark bottles without headspace at room temperature before being analysed within six months from production.

2.2. GC determination of total fatty acid

Fatty acid composition was determined according to Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, and Lercker (2006), as methyl esters by capillary gas chromatography (GC), equipped with a flame ionisation detector (FID), after alkaline treatment. The results were expressed as area normalisation in percent (%). Fatty acids were also reported according to their unsaturation degree, as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. All solvents used were analytical or HPLC grade (Merck, Darmstadt, Germany). Reagents were purchased from Sigma–Aldrich (St. Louis, MO). The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN). Three replicates were prepared and analysed *per* sample.

2.3. DSC

Oil samples (8–10 mg) were weighed into aluminium pans, covers were sealed into place and the whole analysed with a DSC Q100 (TA Instruments, New Castle, DE). Indium (melting temperature 156.6 °C, $\Delta H_f = 28.45\ J\ g^{-1}$) and *n*-dodecane (melting temperature −9.65 °C, $\Delta H_f = 216.73\ J\ g^{-1}$) were used to calibrate the instrument and an empty pan was used as reference. Oil samples were equilibrated at 30 °C for 3 min and then cooled at −80 °C at the rate of 2 °C min^{-1}, equilibrated at −80 °C for 3 min and then heated from −80 to 30 °C at 2 °C min^{-1}. Dry nitrogen was purged in the DSC cell at 50 $cm^3\ min^{-1}$. Thermograms were analysed with Universal Analysis Software (Version 3.9A, TA Instruments) to be exported in an ASCII compatible format. Three replicates were analysed *per* sample.

2.4. Data processing and calibration models

Data were processed employing MVC1 (multivariate calibration 1) routines written for Matlab (Mathworks Inc., Natick, MA, USA), as previously reported (Olivieri, Goicoechea, & Iñón, 2004). PLS models were computed over an overall calibration set of samples for all parameter. Data were mean-centred (MC) before all calculations. Multiplicative signal correction (MSC) pre-treatment was used when necessary considering that MSC performs best if an offset correction is carried out first, as already reported (Isaksson & Næs, 1988). The signal correction was done considering the whole thermogram and providing that all the samples appear to have the same signal level as the ideal. As an estimate of the ideal sample, the average of the calibration was used.

About 30% of the samples was randomly selected as an external validation set for parameter determination, whilst the rest were used to build up the calibration model. The replicates were individually used to take account the sample-to-sample variation in the calibration model.

3. Results and discussion

3.1. Fatty acid composition of oils

Main fatty acid ranges for vegetable oils analysed in this study are reported in Table 1. Among oil samples, 58 were extra virgin olive oil and they were achieved from the two major European country producers (Italy and Spain), whereas the others were obtained from olives growth in Tunisia, which is actually referred as the most important extra-European olive oil producer. All extra virgin olive oil fell within the main fatty acid range composition indicated by the Commission Regulation for this olive oil category (EC, 2007), with palmitic and oleic acids accounted for about 85% of the total fatty acid percentages. The amount of linolenic acid was always lower than 1%, as generally observed for olive oils, whereas content of other minor fatty acids as myristic, arachidic, behenic and lignoceric acids was in accordance with in the range indicated by Commission Regulation for this olive oil category (EC, 2007).

Among samples, Italian extra virgin olive oils were representative of the national oil market, coming from the most important fruit varieties (i.e. Leccino, Coratina) or from the local market. Fatty acid composition was in agreement with previous work (Chiavaro et al., 2010) and presented oleic acid content in the range 64–75% with linoleic acid from 7% to 14%, accounting an oleic/linoleic acid (O/L) ratio higher than seven in most of the samples; this last is considered as the limit value to be overcame for oil oxidative stability. Tunisian virgin olive oils, coming from the most important varieties widespread on the country (Chemlali and Chetoui), were characterised by significantly higher and lower value of linoleic

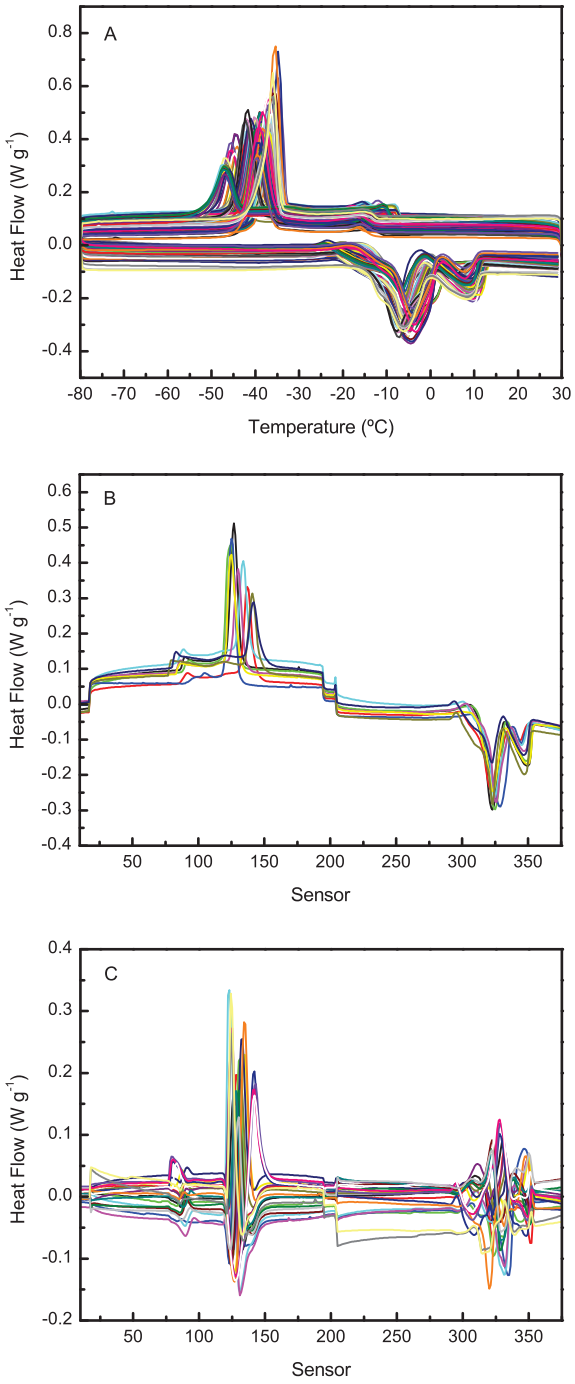

Fig. 1. DSC thermograms (A) obtained; (B) unfolded and (C) mean centred unfolded from selected oil samples.

(~16%) and oleic (~58%) acids, in comparison with the Italian samples, with Chemlali also presenting high palmitic acid amounts (~20%), as previously observed (Baccouri et al., 2008). Spanish samples were obtained from Picual and presented typical composition of oils obtained from this cultivar, similar to those of Italian samples (Allouche, Jiménez, Gaforio, Uceda, & Beltrán, 2007).

Five different oil samples were considered in this work; two (olive oil and olive–pomace oil) were from other commercial categories than extra virgin olive oils, as legally defined by the EC Council of Regulation (2001). The other three (high oleic sunflower, hazelnut and canola oils), having a different botanical origin, were chosen on the basis of their composition rich in oleic and/or linoleic acid, as well as for their common employment in the sector of edible oils.

Olive oil exhibited a fatty acid composition similar to those of extra virgin olive oil samples. On the other hand, olive–pomace oil showed significantly greater linoleic and linolenic acid contents as well as PUFA amount, in comparison to oils directly obtained by olives. This is probably ascribable to their more efficient recovery from seeds due to the application of solvent extraction for oil production (Contiñas, Martínez, Carballo, & Franco, 2008). High oleic sunflower oil presented a higher oleic acid (~80%) and lower palmitic acid (~4.0%) contents that leads to lower SFA and higher MUFA than extra virgin olive oil samples (O'Brien, 2004). Canola oil showed lower oleic (~63%) and higher linoleic (~18%) and linolenic (~8%) acid contents than olive oils, with an higher content of PUFA, as consequence (O'Brien, 2004). Hazelnut oil exhibited a fatty acid composition similar to that of high oleic sunflower oil with an higher contents of palmitic (~6.0%) and linoleic (~11.5%) acids, as previously observed (Chiavaro, Vittadini, Rodriguez-Estrada, Cerretani, & Bendini, 2008).

3.2. DSC analysis of thermograms

DSC is a suitable technique to characterise such phase transitions as crystallisation and melting of vegetable oils that require the intake or release of thermal enthalpy. All DSC thermograms, obtained upon cooling and heating of oil samples, are reported in Fig. 1A. Generally, cooling curves are more interpretable than those obtained upon heating where the well known melting-re-crystallisation phenomenon, named polymorphism, could easily occur for the original oil crystals (Che Man & Tan, 2002; Tan & Che Man, 2002). In addition, crystallisation of oils was well known to be influenced by chemical composition whereas the initial crystalline state has no role in developing transition (Che Man & Tan, 2002).

Extra virgin olive oil samples exhibited two well defined exothermic events, a major one peaking at ~−40 °C and a minor at higher temperature (~13 °C), as previously reported (Chiavaro et al., 2007, 2008, 2010; Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008), with slight shifts of both peaks ascribable to differences of fatty acid composition for some samples (Chiavaro et al., 2010). Similar cooling profiles were also shown by olive and olive–pomace oils although the major peak shifted towards lower temperature, peaking at ~−45 °C, for this latter oil, as previously observed (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008), due to higher unsaturation degree of lipids.

Otherwise, different cooling profiles than those of oils obtained by olive were shown by the seed oils analysed. High oleic sunflower oil, which phase transitions upon DSC were, for the first time, reported in literature in a previous work (Chiavaro et al., 2009), exhibited a single well defined exothermic event peaking at −34 °C with a small shoulder onsetting at ~−15 °C. Crystallisation also occurred over a narrower range of temperature than olive oils being highly cooperative, due to the high content of oleic acid (Chiavaro et al., 2009). Similar cooling profile was exhibited by hazelnut oil with a first, less defined shoulder peak, onsetting at −15 °C, followed by a more important event peaking at −37 °C (Che Man & Tan, 2002; Chiavaro et al., 2008) and by canola oil where a major exothermic event, sharp and tall, peaked at −45 °C and a minor shoulder event was distinguishable at higher temperature (−21 °C) (Che Man & Tan, 2002). These DSC profiles are characteristic of vegetable oils with higher content of oleic and/or linoleic acids than olive oils (Che Man & Tan, 2002; Chiavaro et al., 2008, 2009).

All oil samples exhibited multiple transitions as heated from −80 to 30 °C. Oils from olive showed, at first, a minor exothermic peak and, successively, a major endothermic event occurring over the −18/12 °C temperature range. A first exothermic event,

Table 1
Statistical summary and figures of merit from the DSC–PLS calibration for all oil parameters.

Calibration	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	O/L	U/S	SFA	MUFA	PUFA
Concentration range (%)	3.9–20.0	1.7–4.1	53.8–80.4	4.7–19.3	2.8–17.5	3.2–40.1	8.2–23.8	56.4–82.8	5.2–26.8
Statistical summary									
PLS factors	13	11	16	10	13	11	10	12	11
Pre-treatment	MC	MC	MC	MC	MC	MC	MC	MC	MC
	–	MSC	–	–	–	–	–	–	–
RMSE	0.92	0.25	1.50	1.05	0.92	0.67	1.03	1.85	1.06
REC	7.21	9.15	2.10	10.80	10.87	11.06	6.35	2.53	10.15
R^2	0.92	0.78	0.94	0.92	0.91	0.98	0.89	0.91	0.94
Figures of merit									
Sensitivity	0.41	1.6	0.15	0.62	0.46	0.4	0.63	0.26	0.53
Analytical sensitivity	9.8	31	4.5	9.9	11	7.5	9.9	5.8	10
Selectivity	0.25	0.31	0.13	0.32	0.2	0.5	0.37	0.23	0.31
LOD (3.3*SD)	0.96	0.24	1.95	1.08	1.01	0.72	0.75	1.96	11.92

Abbreviations used: O/L, ratio between oleic and linoleic acid percentages; U/S, ratio between unsaturated and saturated fatty acid percentages; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; MC, mean-centre; MSC, multiplicative scatter correction; RMS, root mean square; REC, relative error in calibration; LOD, limit of detection.

occurring in the −30 to −15 °C temperature range, was distinguishable for extra virgin oils and olive oil sample but less evident in olive–pomace oil, as it was spread over a larger temperature range (from −40 to −15 °C) (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008). It was previously attributed to an exothermic molecular rearrangement of crystals into more stable polymorphic forms (Tan & Che Man, 2002). On the other hand, the complex endothermic events occurring at higher temperatures were related to the melting of crystallised lipids and characterised by multiple overlapping contributions (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008; Tan & Che Man, 2002). In particular, two endothermic events were more distinguishable. The major peaked at lower temperature, in the range −3.5/−7.0 °C for oil samples from olive, probably in relation with the unsaturation degree of lipid (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008). Additional endothermic events were possibly observed in some samples, displaying themselves as shoulder peaks embedded in the major event at lower or higher temperature (at ∼−14 and ∼−3 °C).

The minor endotherm peaking at higher temperature (in the range 6.0/8.0 °C), showed different peak profiles among samples, being more evident and wider, with a maximum skewed towards higher temperature, or sharper and more symmetric or still hardly distinguishable. These differences were previously observed for extra virgin olive oil samples of different lipid composition in relation to cultivar and/or commercial categories (Chiavaro, Rodriguez-Estrada, Barnaba et al., 2008).

High oleic sunflower oil exhibited a heating profile with a well distinct endothermic event peaking at about −7 °C and a shoulder peak embedded in the major one peaking at higher temperature (about −2.5 °C), as previously reported (Chiavaro et al., 2009). Heating thermogram of hazelnut oil sample showed an endothermic event peaking at −7.0 °C, with two less distinct shoulder peaks, at lower (∼−15 °C) and higher temperatures (∼−2 °C) (Chiavaro et al., 2008; Tan & Che Man, 2002). Heating transition of canola oil displayed itself with a broad endotherm formed by four overlapping transitions; the major one peaked at about −16 °C, whereas the minor transitions were set at lower (about −26 and −22 °C, respectively) and higher (about −9 °C) temperatures, as previously observed (Tan & Che Man, 2002). All these profiles are related to the melting of different polymorphic forms of lipid crystals probably due to the kind of acyl moieties linked to glyceride skeleton.

3.3. PLS model construction for calibration and validation

The vegetable oils analysed in this study exhibited different substitution patterns, also differing in the chain length and position of the acyl moieties, as well as in their unsaturation degree, as above described. Thus, samples of oils from other botanical origin as well from different commercial categories were added to extra virgin olive oils to construct a more robust model for calibration, enlarging ranges of fatty acid composition.

A multivariate approach was used to obtain practical information to quantitatively approach DSC data, as differences in the DSC thermograms ascribable to fatty acid composition are not easily and always detectable by univariate analysis. In particular, PLS, a full data method, was proposed as possible solution, and it was applied to unfolded DSC data (Fig. 1B).

In order to predict the main fatty acids (palmitic, stearic, oleic and linoleic acids) and their percentage sums, (SFA, MUFA and PUFA) as well as quality ratios (O/L and U/S ratios) in olive oil, several multivariate calibration models were built by the PLS algorithm, using the pre-processed spectral data reported in Table 1 where the statistical summary and figures of merit from the DSC–PLS calibration for all oil parameters are also reported. The appropriate number of model dimensions, which was individually found out for each quality parameter, was determined applying the Haaland and Thomas statistical criterion ($\alpha = 0.75$) (Haaland & Thomas, 1988).

The best prediction ability was achieved when the PLS calibration was carried out on the mean centred (MC) unfolded DSC data of oil samples (Fig. 1C). In addition, for stearic acid also MSC pretreatment was necessary to achieve good results. Acceptable values were obtained for RMSE (root mean square error) and REC% (percentage relative error in calibration). These parameters measure the average error in the analysis and evaluate the goodness of fit of the calibration data to the models developed during calibration. LODs (limit of detection) under linear minimum calibration concentration were found, indicating that the linear range is able to quantification. R^2, which describes the goodness of fit of the predicted concentrations to their actual values, was higher than 0.90 for almost all parameters. A 0.78 R^2 value was found for stearic acid and this may be due to its narrow range of composition. The figures of merit demonstrated the quality of the models and the suitability of the method for the proposed determinations.

DSC–PLS statistical results for the prediction of selected parameters in the validation set are reported in Table 2. The validation set exhibited nearly quantitative recoveries (between 98.2% and 103.4%) for all calibrated parameters. Relative standard deviations below 7% were found for palmitic and oleic acids as well as for SFA whereas stearic and linoleic acids as well as MUFA and PUFA and U/S ratio exhibited relative standard deviations slightly higher but lower than 15%.

The yields obtained are illustrated in Fig. 2, showing a good agreement between predicted and actual levels on validation data

Table 2
DSC–PLS statistical results for the prediction of selected parameters in the validation set. Standard deviation is given in parentheses.

Validation	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	O/L	U/S	SFA	MUFA	PUFA
RMSE (conc. units)	0.878	0.248	2.232	0.995	1.263	0.584	0.986	1.753	1.040
REP (%)	6.89	9.15	3.13	10.25	14.87	9.63	6.07	2.39	9.95
R^2	0.936	0.776	0.901	0.947	0.843	0.838	0.906	0.939	0.944
Recovery (%)	100.0 (6.9)	103.0 (10.2)	100.6 (3.2)	100.1 (12.2)	103.4 (16.4)	100.9 (11.8)	99.6 (5.7)	98.2 (13.6)	100.4 (11.7)
Slope	0.94 (0.03)	0.80 (0.06)	0.93 (0.04)	0.95 (0.03)	0.91 (0.06)	0.95 (0.06)	0.91 (0.04)	0.91 (0.03)	0.93 (0.03)
Intercept	0.75 (0.46)	0.59 (0.16)	5.52 (2.99)	0.51 (0.34)	0.85 (0.50)	0.32 (0.32)	1.40 (0.70)	6.13 (2.38)	0.36 (0.36)

Abbreviations used: O/L, ratio between oleic and linoleic acid percentages; U/S, ratio between unsaturated and saturated fatty acid percentages; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; RMSE, root mean square error; REP, relative error in prediction.

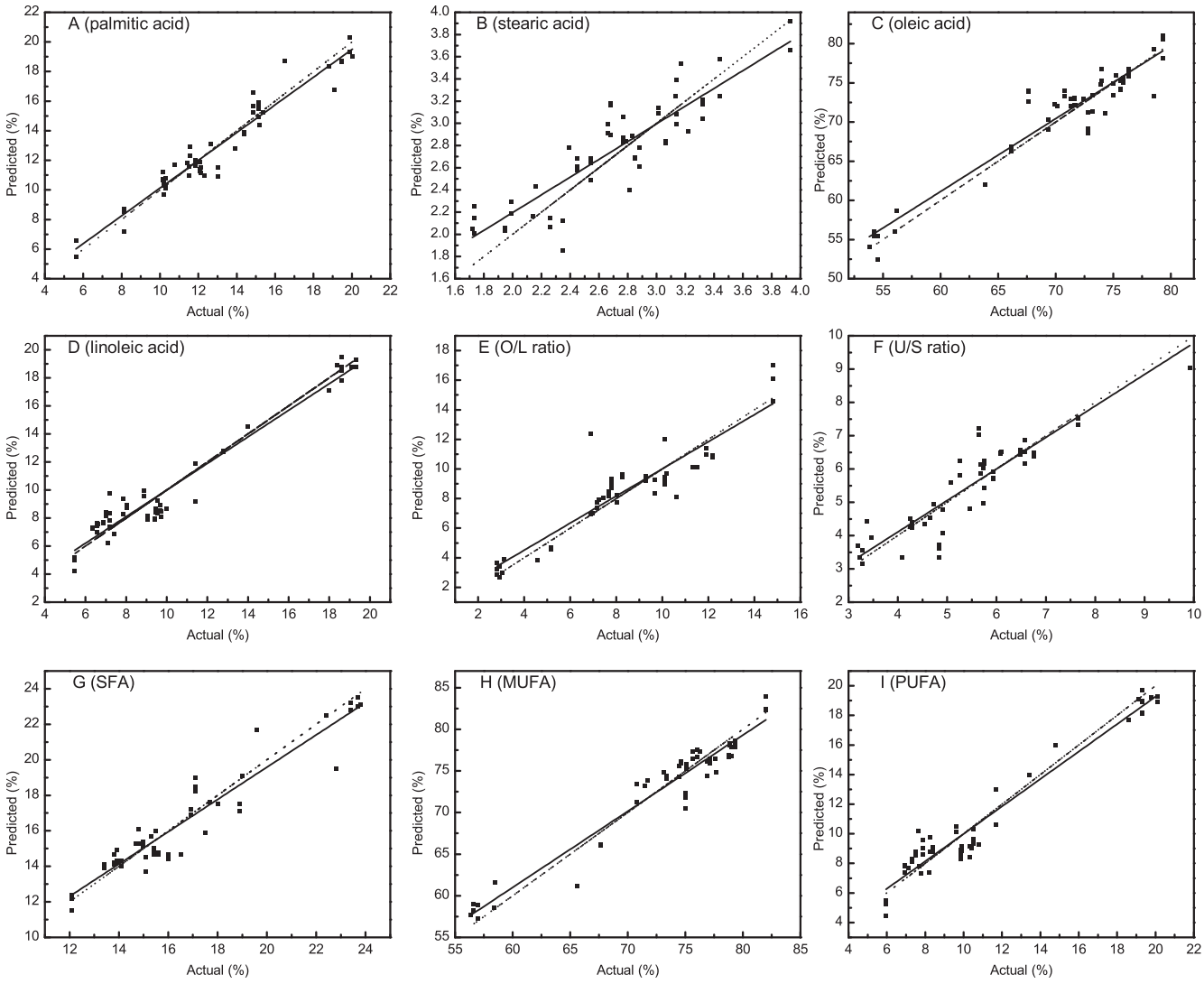

Fig. 2. Actual vs. DSC–PLS predicted values in the validation (■) sets for (A) palmitic acid, (B) stearic acid, (C) oleic acid, (D) linoleic acid, (E) O/L ratio, (F) U/S ratio, (G) SFA, (H) MUFA, (I) PUFA. Ideal fitting (intercept = 0, slope = 1, —) and actual fitting curves (- -) for predicted values of (A) palmitic acid, (B) stearic acid, (C) oleic acid, (D) linoleic acid, (E) O/L ratio, (F) U/S ratio, (G) SFA, (H) MUFA, (I) PUFA are shown.

sets. The fitting curves of actual vs predicted values are close to ideal fitting curve, slope and intercept equal to unity and zero, respectively, indicating low bias and absence of systematic regression errors (Table 2).

4. Conclusions

In this work, a novel strategy based on DSC–PLS for the determination of fatty acid composition of olive oil was developed, presenting an approach that was little or not present in literature jet and very suitable for the quality evaluation of the most precious vegetable oil. In particular, the model appeared to be suitable for the determination of overall, oleic acid content, which is related to oil health benefits. Results are also excellent for palmitic acid and SFA contents, showing very good correlation coefficients and low RMSE values in both calibration and validation sets. Satisfactory results were also obtained for MUFA, PUFA, stearic and linoleic acids, and O/L ratio in terms of percentage recoveries and relative

standard deviations. These good findings were obtained considering a large set of olive oil samples representative of the European market with the addition of few samples of different botanical provenience to enforce the robustness of the model, evidencing the capability of PLS based analytical procedures for simultaneously processing information obtained from different analytical signals as well as the discriminatory power resulted by DSC data.

In conclusion, this novel approach could provide results statistically similar to traditional official procedures in terms of analytical performance being very rapid and environmentally friendly and also proposing itself as a suitable tool for quality assurance besides than research purposes.

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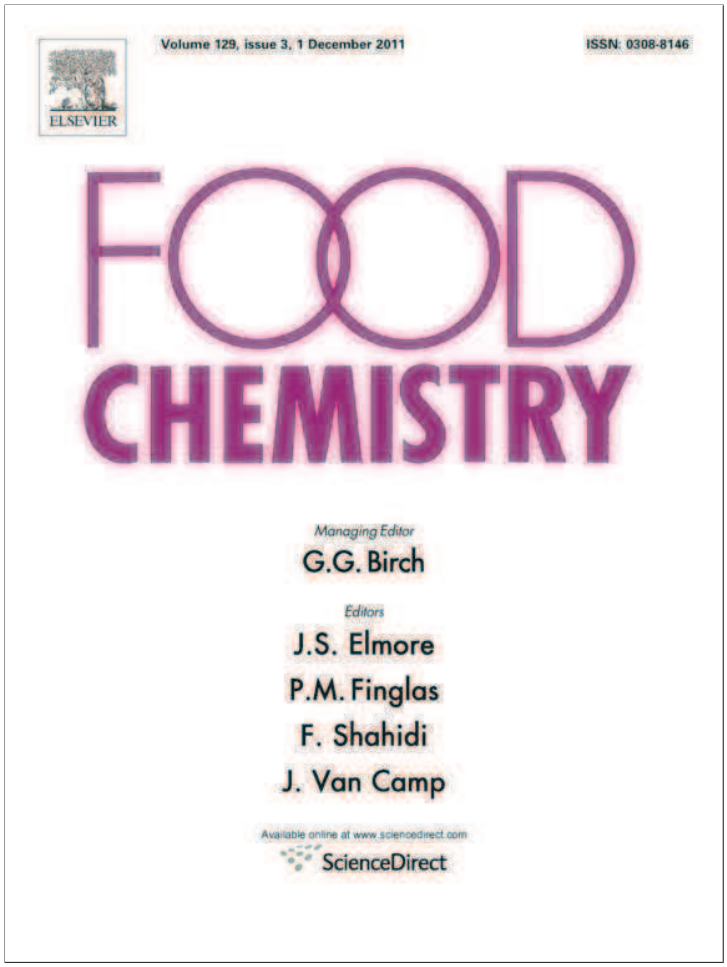
ARTICOLO 6

Cevoli, C., **Cerretani, L.**, Gori, A., Caboni, M.F., Gallina Toschi, T., Fabbri, A. Classification of Pecorino cheeses using electronic nose combined with artificial neural network and comparison with GC-MS analysis of volatile compounds. *Food Chemistry* 129 (3), pp. 1315-1319, 2011 (IF 3,655).



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Analytical Methods

Classification of Pecorino cheeses using electronic nose combined with artificial neural network and comparison with GC–MS analysis of volatile compounds

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ABSTRACT

An electronic nose based on an array of 6 metal oxide semiconductor sensors was used, jointly with artificial neural network (ANN) method, to classify Pecorino cheeses according to their ripening time and manufacturing techniques. For this purpose different pre-treatments of electronic nose signals have been tested. In particular, four different features extraction algorithms were compared with a principal component analysis (PCA) using to reduce the dimensionality of data set (data consisted of 900 data points per sensor). All the ANN models (with different pre-treatment data) have different capability to predict the Pecorino cheeses categories. In particular, PCA show better results (classification performance: 100%; RMSE: 0.024) in comparison with other pre-treatment systems.

1. Introduction

Pecorino is a generic name that indicates Italian cheeses made only from raw or thermized sheep's milk; the cheese is produced almost exclusively in the middle and south of Italy according to ancient and unique manufacturing techniques. Due to the wide range of such cheeses in Italy, it is very difficult to group them under a single class, in fact some variables (such as production area, pastures, cheese processing techniques and the degree of ageing) can influence the diversity of local productions and consequently affect flavour (Coda et al., 2006; Grappin & Beuvier, 1997; Ziino, Condurso, Romeo, Giuffrida, & Verzera, 2005). Some Pecorino cheeses have recently gained the prestigious denomination 'PDO' (Protected Denomination of Origin) (such as Pecorino Sardo, Pecorino Romano, Pecorino Toscano, Pecorino Siciliano, Fiore Sardo, Canestrato Pugliese), although in Italy there are a large number of Pecorino cheeses without a designation of origin. Several authors investigated the nature of the flavours in the Italian and Iberian PDO Pecorino cheeses (Barron et al., 2007; Delgado, González-Crespo, Cava, García-Parra, & Ramírez, 2010; Di Cagno et al., 2003; Izco & Torre, 2000; Larráyoz, Addis, Gauch, & Bosset, 2001; Martinez-Castro, Sanz, Amigo, Ramos, & Martin-Alvarez, 1991; Ortigosa, Torre, & Izco, 2001; Pirisi, Achilleos, Jaros, Noel, & Rohm, 2000; Villasenor, Valero, Sanz, & Martìnez-Castro, 2000) by microbiological, compositional, biochemical, volatile profile and sensory analysis; different compounds of the volatile and non-volatile fraction that could give rise to different flavours have been identified and related to specific processes from which they may be generated.

Recently, electronic nose have been shown to be valid instruments that are applicable in many fields of food for the aroma control; these sensors have a low cost and can work on-line without sample pretreatment (Berna, 2010).

An electronic nose is an instrument, which comprises an array of electronic chemical sensors (often referred as gas sensor array) with partial specificity and an appropriate pattern-recognition system, capable of recognising simple or complex odours (Gardner & Bartlett, 1993).

The electronic nose has been successful for classification and prediction purposes by monitoring the flavour of several diary products (Ampuero & Bosset, 2003; Peris & Escuder-Gilabert, 2009; Schaller, Bosset, & Escher, 1998a, 1998b). Particularly, the studies on the electronic nose applications to cheese are relative to classification by the cheese variety (Contarini et al., 2001; Jou & Harper, 1998), geographical origin (Pillonel, Ampuero, Tabacchi, & Bosset, 2003; Pillonel et al., 2003), cheese ripening stage (Contarini et al., 2001; Schaller, Bosset, & Escher, 1998a, 1998b; Trihaas & Nielsen, 2005) and to shelf life determination (Benedetti, Sinelli, Buratti, & Riva, 2005).

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The processing of data generated by a gas sensor array represents a crucial part of the electronic nose concept, as the multivariate output signal generated may often be non-linear in nature. A variety of pattern recognition techniques have been utilised such as multivariate analyses (principal component analysis PCA, canonical discriminant analysis CDA, feature weighting and cluster analysis CA) graphical analyses (bar chart, profile, polar and offset polar plots and network analyses (artificial neural network ANN and radial basis function RBF) (Sohn, Smith, Yoong, Leis, & Galvin, 2003). Several authors have observed that ANNs can provide higher classification and prediction probability than conventional multivariate analysis for complex non-linear data (Brezmes, Ferreras, Llobet, Vilanova, & Correig, 1997; Gardner & Bartlett, 1993; Paliwal, Visen, Jayas, & White, 2003; Ping & Jun, 1996; Schaller, Bosset, & Escher, 1998a, 1998b; Ushada & Murase, 2006; Winquist, Hornsten, Sundgren, & Lundstrom, 1993).

The aim of the present study was to develop a rapid method, based on MOS sensors (metal oxide semiconductor), capable of classifying pecorino cheese according to their ripening time and manufacturing techniques. For this purpose electronic nose signals were pre-treated by different features extraction algorithms or by PCA and the capability of classification of these pre-treatment systems have been tested by ANN method. Moreover, the results of the proposed method were compared to those obtained analysing the GC–MS data by ANN.

2. Materials and methods

2.1. Cheese samples

Cheese samples were kindly obtained from the 6th edition of the national concourse "*Ovillus Aureus, the Gold Pecorino Cheese*" (November 28th, 2008, Fano, Italy) reserved for typical Italian Pecorino and "fossa" Pecorino cheeses. The typical feature of Fossa cheese concerns the seasoning in flask-shaped pits which are dug in the tufa soil of Sogliano al Rubicone (Emilia-Romagna region). This annual competition aims to promote typical and quality dairy production in Italy, and to emphasise the qualitative and organoleptic characteristics of Italian Pecorino cheeses.

Samples were grouped into three categories: 28 hard cheeses (aged more than 6 months) labelled hard "non-fossa", 14 semi-hard cheeses (aged for 2–6 months) labelled semi-hard "non-fossa", and 18 Fossa Pecorino cheese (aged from 3 to 8 months) labelled hard "fossa". Cheeses were packed and stored at −40 °C at our laboratories until analyses. The categories and the geographical origins were guaranteed by the supplier.

2.2. Analysis of aroma by electronic nose

2.2.1. Instrumentation and working conditions

An electronic olfactory system (EOS 507, Sacmi Imola S.C., Imola, Bologna, Italy) composed of a measuring chamber with 6 metal oxide sensors and a personal computer was used for the acquisition and analysis of the data generated by the EOS 507. The sensors used were: sensor 1 (SnO_2), sensor 2 ($SnO_2 + SiO_2$), sensor 3, 4 and 5 (catalysed SnO_2 with Au, Ag and PD, respectively) and sensor 6 (WO_3). During the analysis, sensors were maintained at a temperature range of 350–450 °C. The EOS 507 was controlled by an integrated PDA equipped with proprietary software, and was connected to an automatic sampling apparatus (Model HT500H) which had a carousel of 10 sites for loading samples. Samples were kept at controlled temperature (37 °C) and placed in a chamber provided by a system that removes humidity from the surrounding environment.

2.2.2. MOS sensor array procedure

For each sample, 15 g were placed in 100 mL Pyrex vials equipped with a pierceable silicon/Teflon cap. For each sensor, signal is divided in three parts (see Fig. 1): (*before*) conditioning phase (25 min period employed to obtain a constant baseline) and before injection phase (in which samples were incubated at 37 °C for 7 min before injection), (*during*) measurement cycle (in which the oil headspace, sampled with an automatic syringe, was then pumped over the sensor surfaces for 2 min during which the sensor signals were recorded; in this phase, sensors were exposed to filtered air at a constant flow rate of 50 sccm (standard cubic cm per min) to obtain the baseline) and (*after*) recovery phase (another 7 min period applied to restore the original MOS conditions). Ambient air filtered with activated silica and charcoal was used as a reference gas during the recovery phase of the measurement cycle. The previous conditions ensured that the baseline reading had indeed been recovered before the next analysis was performed. The experimental conditions adapted from Camurati, Tagliabue, Bresciani, Sberveglieri, and Zaganelli (2006) were used.

2.3. Analysis of volatile compounds

2.3.1. Samples preparation

Prior to analysis cheese samples were reduced into small granules using a ceramic mortar and pestle; 15 g of each cheese were then placed in a 100 mL bottle and closed with a silicon cap. After 40 min pre-heating in a 40 °C water bath, the cap was perforated with a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, coating 2 cm) fibre holder (Supelco Ltd., Bellefonte, PA, USA) and incubated for 30 min in a heating bath at 40 °C. Finally, the fibre was exposed in the injector, and immediately desorbed for 5 min at 240 °C in the gas chromatograph.

Volatile compounds were tentatively identified basing on computer matching against commercial libraries (NIST/EPA/NIH Mass Spectral Library 2005) as well as our laboratory-made spectral library of pure substances, Kovats retention indices (KI) and literature data. Retention indices were calculated for each compound using homologous series of C9–C19 *n*-alkanes.

2.3.2. GC–MS volatile analysis

The determination of volatile components was performed in triple by solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME/GC–MS). Volatile compounds were identified by gas chromatography coupled to quadrupolar mass-selective spectrometry using an Agilent 6890 N Network gas chromatograph and an Agilent 5973 Network detector (Agilent

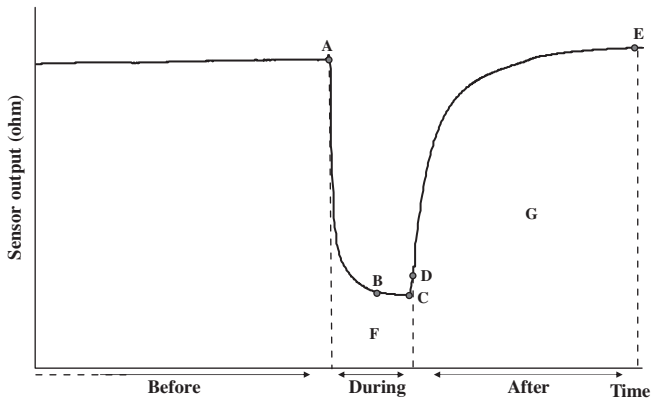

Fig. 1. Plot representing the electric resistance of aMOS sensor during Pecorino cheese evaluation: (*before*) injection phase, (*during*) measurement cycle and (*after*) recovery phase.

Technologies, Palo Alto, CA, USA). Analytes were separated on a ZB-WAX Phenomenex column (30 m × 0.25 mm ID, 1.00 μm film thickness). Column temperature was initially held at 40 °C for 10 min. For the first ramp, the temperature was increased to 200 °C at 3 °C min^{-1} and then held for 3 min, while in the second to 250 °C at 10 °C min^{-1} for 3 min. The ion source and the transfer line were set to 230 °C and 150 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionisation energy in the 20–250 amu mass range (2 scan/s). For each samples we recorded the presence or absence of individual volatile compound for following ANN analysis.

2.4. Nose signal pre-treatment

2.4.1. PCA analysis

The main use of PCA is to reduce the dimensionality of data set while retaining as much information as possible. It computes a compact and optimal description of the data set, by means of the principal components (PCs), that are new uncorrelated variables calculated to replace the original data. The first significant component explains the largest percentage of the total variance, the second one, the second largest percentage, and so forth. There can be as many possible PCs as variables [32].

The acquired gas sensor data consisted of 900 data points per sensor. This large data file could drive to very high calculation time if directly used as input in an ANN. Consequently the variables were compressed by PCA to get the most significant PCs scores, using CAMO Software (Unscrambler 9.7, CAMO Software Oslo, Norway).

Generally, when the PCs have more than 85% cumulated reliability of the original dataset, these PCs can be used to replace the original one. However, in practical application, the value 85% is not always necessary to be achieved, and the number of PCs can be changed to some extend according to the circumstance (He, Feng, Deng, & Li, 2006) In this study a cumulated reliability threshold of 98% was used. In Table 1 the weight for the several PCs of different sensor are shown. Nineteen PCs was selected and their scores were used as ANN input variables.

2.4.2. Software features

The data from the electronic nose was even analysed with the statistical package "Nose Pattern Editor" (Sacmi Imola S.C. v.5.5.0). Different features extraction algorithm named *Classical* (F1), *Classical_after* (F2), *Phase_integral* (F3) and *Last_diff* (F4), were applied to the data before neural network treatment.The response extracted by each sensor were defined by:

$$F1 = C/A$$
$$F2 = D/E$$
$$F3 = F + G$$
$$F4 = D - B$$

where A is the last *before* step point, B the central *during* step point, C the minimum *during* stet value, D the last *during* step point, E the last *after* step point, F the area *during* step and G the area *after* step (see Fig. 1).

Table 1
Cumulative reliability of principal components (%) of 6 sensors considering a threshold of 98%.

Principal component	Cumulative reliability %					
	Sensor 1	Sensor 2	Sensor 3	Sensor 4	Sensor 5	Sensor 6
PC1	82.87	91.23	83.36	89.19	79.83	95.26
PC2	92.78	99.21	93.46	97.22	92.76	98.60
PC3	96.66	–	97.11	99.36	96.49	–
PC4	98.40	–	98.85	–	98.69	–

2.5. ANN models

ANN models were constructed using STATISTICA Neural Networks 4.0 (StatSoft Inc., Tulsa, OK, USA). ANN is a mathematical algorithm which has the capability of relating the input and output parameter, learning from examples through iteration without requiring a prior knowledge on the relationships between the process variables (Smith, 1996).

The neural network model used in this study was a multilayer perceptron (MLP) that learns using an algorithm called back propagation with an adaptive learning rate and momentum of 0.1 and 0.8 respectively.

For each typology of neural network, for the input and hidden layers were used a linear and a logistic activation function, respectively, while the softmax function was used for the output layer. From a statistical point of view, with the softmax activation function and the cross-entropy error, the neural network model can be seen as a multilogistic regression model, and then the outputs are interpretable as posterior probabilities for the categorical target variables (Bishop, 1995).

Three ANN schemes characterised by different input layer, but by the same output layer were tested. The input values were represented or by scores of 19 PCs previously selected (19 input neurons) or by the different features extracted (F1, F2, F3 and F4) for each sensor (6 input neurons) or by the results of GC–MS volatile analysis (184 input neurons). Three nominal output variables (V_1, V_2 and V_3) were used to perform classification tasks: V_1 for the hard "fossa" samples V_2 for the hard "non-fossa" samples and V_3 for the semi-hard "non-fossa" samples, assuming that the target output is 1.0 in the correct class output, and 0.0 in the others. A useful interpretation of the output units is that of probabilities. The outputs can be regarded as estimates of the probability of membership of each class, provided that we ensure that the total across all outputs is 1.0.

The original dataset was randomly divided into *training set* (60%), *verification set* (20%) and *test set* (20%). The *verification set* was used to identify the best network on the basis of the network's error performance and to stop *training* if over-learning occurs; the *test set* was performed to give an independent assessment of the network capability of classifying Pecorino cheeses samples.

It is important to determine the number of neurons in the hidden layer in order to build a good ANN. The ANN with too many hidden neurons can show low training error, but high generalisation error due to overfitting (He et al., 2006). A number of hidden neurons between the input and output layer size is recommended and generally is empirically determined (Berry & Linoff, 1997; Boger & Guterman, 1997; Swingler, 1996). Networks with one hidden layer, but with different number of neurons were tested to select the most accurate classification results.

3. Results and discussion

An early stopping technique was used to get good generalisation performance and to decrease the number of epochs. The error on the verification set is monitored during the training process, controlling the total training process of an ANN. The verification error normally decreases during the initial phase of training, as does the training set error.

However, when the network begins to overfit the data, the error on the verification set typically begins to rise. When the verification error increases for a specified number of iterations, the training is stopped (Demuth & Beale, 1994).

Fig. 2 details a plot of the root mean square error (RMSE) relative to verification data and training data of a ANN with 19 input units (scores of PCs).Results of *training*, *verification* and *test* of the neural networks with 19 input unit are summarised in Table 2.

With the increase in the number of neurons in the hidden layer, a minimal decrease in the root mean square error of test set was observed. However, no improvement in the ANN performance was observed. In particular, from *test* validations, 100% of the data set is correctly classified for each neural networks tested.

For the samples of hard "fossa" Pecorino cheese, the lowest probability of correct identification is about 0.965, for the samples of hard "non-fossa" Pecorino cheese is about 0.967 and for the semi-hard "non-fossa" Pecorino cheese samples is about 0.919.Results of *training*, *verification* and *test* of the best neural networks with 6 input unit (feature) are summarised in Table 3.

All performed MLP neural networks appear to classify the data test samples in the same way, but the result (72%) appears rather low. The higher probability of correct identification of the all data set samples is related to F2 (about 0.66). The test set root mean square errors are considerably greater than those related to neural network with 19 input units (PCs).

The above results reveal that an important part of information contained in the nose signal was lost using the features as input. In fact the percentage of correct classification cases is substantially high for the ANN built with the PCs score as input. The PCA analysis was an efficient way to reduce the dimensionality of the original data set. The computation time of the two ANN type resulted similar and lasting a few minutes.Results of *training*, *verification* and *test* of the best neural networks with 184 input unit (GC–MS) are summarised in Table 4.

With the increase in the number of units in the hidden layer, a decrease in the root mean square error was observed, but not an increase in the classification accuracy. In particular, from *test* validations, 86% of the data set is correctly classified for each neural networks tested with more than 5 units in the hidden layer.However, no improvement in the RMSE (test set) and ANN performance were observed in neural network with more than 35 units in the hidden layer.The root mean square errors are notably greater than those related to neural network with 19 input units (PCs).In this case the computation time resulted much higher, approximately 30 min.

In general appeared that the best results were obtained by the e-nose signals elaborations. This is supported by the fact that SPME–GC–MS can identify only individual chemical compound while the electronic nose can recognise even the combination of individual chemical compound in the generation of odours. Moreover, the analysis by electronic nose resulted to be time consuming than those carried out by SPME–GC–MS, and easily applied for real time quality control.

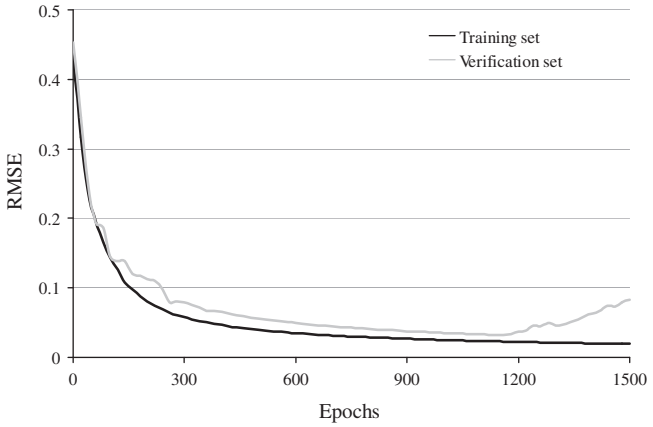

Fig. 2. Plot of root mean square error (RMSE) history relative to ANN with 19 input units (PCs scores) and 4 units in the hidden layer.

Table 2
Results of ANN with 19 input units (PC scores): correctly classified cases (%), test set root mean square error (RMSE) and hidden units number.

Hidden units	RMSE	Correct classification cases (%)		
		Training	Verification	Test
2	0.028	100	100	100
3	0.027	100	100	100
4	0.025	100	100	100
10	0.024	100	100	100

Table 3
Results of ANN with 6 input units (feature extraction): correctly classified cases (%), test set root mean square error (RMSE) and hidden units number.

Feature	Hidden units	RMSE	Correctly classification cases (%)		
			Training	Verification	Test
F1	4	0.409	95	86	72
F2	4	0.347	95	72	72
F3	3	0.459	86	72	72
F4	3	0.432	86	72	72

Table 4
Results of ANN with 184 input units (results of GC–MS volatile analysis): correctly classified cases (%), test set root mean square error (RMSE) and hidden units number.

Hidden units	RMSE	Correct classification cases (%)		
		Training	Verification	Test
5	0.385	100	100	72
10	0.342	100	86	86
20	0.325	100	86	86
30	0.233	100	86	86
35	0.222	100	86	86

A comparison with other investigations (Contarini et al., 2001) on the analysis by electronic nose of Pecorino cheeses which differed in their maturation time, raised that our results are very promising. In particular, Contarini et al. (2001) using electronic nose and the GC–MS to discriminate two different Pecorino cheese, reported that the two group were not widely separated by both techniques.

4. Conclusions

For the considered samples the classification capability of ANN and e-nose coupling were higher than the technique based on GC–MS analysis of volatile compounds.

The results have shown how the ANN and e-nose coupling can be a very effective classification technique for Pecorino cheese, easy to implement and not computationally expensive, even if needs to be well understood and set up by researcher.

It is always possible to consider the entire dataset, accepting an higher computational effort, even if the small loss of information due to PCs extraction make possible to reduce calculation time without significant precision loss. On the contrary, data feature extraction was not a successfully technique.

Acknowledgements

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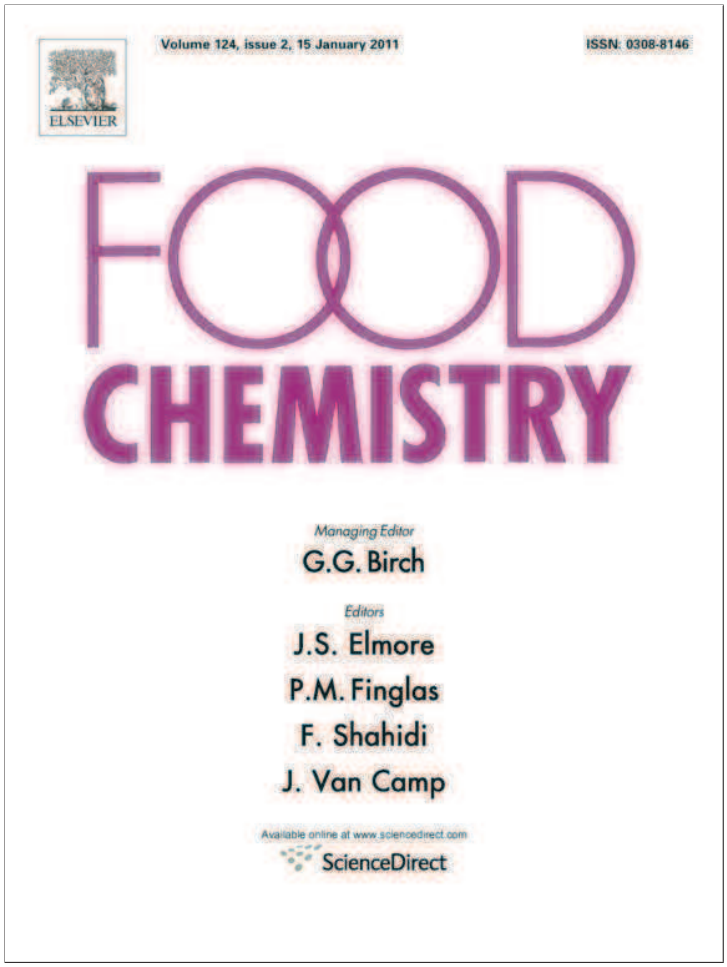
ARTICOLO 7

Lerma-García, M.J., Simó-Alfonso, E.F., Bendini, A., **Cerretani, L.** Rapid evaluation of oxidised fatty acid concentration in virgin olive oil using Fourier-transform infrared spectroscopy and multiple linear regression. *Food Chemistry* 124 (2), pp. 679-684, 2011 (IF 3,655).



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Analytical Methods

Rapid evaluation of oxidised fatty acid concentration in virgin olive oil using Fourier-transform infrared spectroscopy and multiple linear regression

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ABSTRACT

Fourier-transform infrared spectroscopy (FTIR), followed by multivariate treatment of spectral data, was used to evaluate the oxidised fatty acid (OFA) concentration in virgin olive oil samples characterised by different oxidative status. The entire FTIR spectra (4000–700 cm^{-1}) of oils were divided in 25 wavelength regions. The normalised absorbances of the peak areas within these regions were used as predictors. In order to predict the OFA concentration, multiple linear regression (MLR) models were performed. After a cube root transformation of data, an MLR model constructed using eight predictors was able to predict OFA concentration with an average error of 17%. The main wavelength regions selected to construct this MLR model corresponded to =C–H (trans and cis, stretching), –C–H (CH_2, stretching asym), O–H (bending in plane), C–O (stretching), –H–C=C–H– (cis?) and =CH_2 (wagging), due to the fact that these regions were those more affected by oxidation. This FTIR method is an extremely quick and simple procedure for OFA determination which can be easily automatised.

1. Introduction

Virgin olive oils (VOOs) are characterised by a high oxidative stability with respect to other edible oils in terms of their fatty acid composition (a high oleic acid concentration) and antioxidant content (Bendini, Cerretani, Carrasco-Pancorbo, et al., 2007). The stability to oxidation is an important indicator to determine both the quality and shelf life (Hamilton, 1994). Peroxide value is one of the most frequently used quality parameters which measures the amount of total peroxides as a primary product of oil oxidation during production, storage and marketing. (Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, & Lercker, 2006).

On the other hand, oil oxidation also produces different types of secondary oxidation products that derive from fatty acids and other minor compounds, such as oxidised fatty acids (OFAs), oxidised polymers and volatile compounds among others (Choe & Min, 2006). The formation of these oxidation products depends on the fatty acid composition and on the oxidation conditions (Al-Ismail, Caboni, & Lercker, 1998; Al-Ismail, Caboni, Rodríguez-Estrada, & Lercker, 1999).

The evaluation of secondary oxidation products represents a critical point to estimate, among others, the storage status of fatty substances (Al-Ismail et al., 1998).

Rovellini, Cortesi, and Fedeli (1998) have proposed a method to evaluate the oxidation status of VOOs using RP-HPLC analysis of OFAs. This method has been used in various areas of applied research for VOO (Armaforte et al., 2007; Bendini et al., 2006; Rovellini & Cortesi, 2004) as well as for the analysis of lipid extracts from other matrices (Verardo et al., 2009).

During the last few years, spectroscopic methods combined with Fourier transformed (FTIR and FTNIR) techniques have been widely applied in the fat and oil sector due to the ability of these techniques to work in close relation to production processes (Bendini, Cerretani, Di Virgilio, Belloni, Lercker, et al., 2007). FTIR methods are fast, simple-to-perform and do not require sample pretreatment. FTIR has been successfully used to establish a number of oil parameters such as acidity (Al-Alawi, van de Voort, & Sedman, 2004; Bertran et al., 1999; Iñón, Garrigues, Garrigues, Molina, & de la Guardia, 2003), peroxide (Bendini, Cerretani, Di Virgilio, Belloni, Bonoli-Carbognin, et al., 2007; Guillén & Cabo, 2002; Li, van de Voort, Ismail, & Cox, 2000), iodine levels (Li, van de Voort, Ismail, Sedman, et al., 2000) and fatty acid composition (Maggio et al., 2009). It has been also used to establish VOO freshness in combination with multivariate analysis to discriminate samples at different periods of storage in dark or light conditions (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007) and to establish VOO authenticity (Lerma-García, Ramis-Ramos, Herrero-Martínez, & Simó-Alfonso, 2009; Marigheto, Kemsley, Defernez, & Wilson, 1998; Yang & Irudayaraj, 2001).

In this work, a rapid FTIR method, jointly with the application of multiple linear regression (MLR) models, is used to evaluate OFA

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concentration in VOOs characterised by different oxidative status. For this purpose, FTIR spectra were divided in 25 wavelength regions, using the normalised absorbance peak areas as predictors.

2. Experimental

2.1. Reagents and samples

Tricaproin, triheptadecanoin, sodium benzyloxide in benzyl alcohol, *n*-hexane, 2-propanol and acetone have been purchased to Sigma-Aldrich (St. Louis, MO, USA), acetonitrile (ACN) and anhydrous sodium sulphate from Merck (Darmstadt, Germany), and acetic acid from Fluka (Buchs, Switzerland).

A series of 72 VOOs were sampled from different Italian regions (Abruzzo, Emilia-Romagna, Puglia, Sicilia and Toscana) during the harvest seasons 2006–2007; 2007–2008 and 2008–2009. All samples were analysed between November 2008 and January 2009. The oils differed in terms of olive cultivar, degree of ripening, area of growth, production system (type, productive capacity and manufacturer) and storage time.

2.2. OFA determination

OFA determination was performed using a 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) provided with a binary pump delivery system, a degasser, an autosampler and a diode array UV–Vis detector (DAD). The liquid chromatograph was also coupled (in series with the DAD) to an atmospheric pressure chemical ionisation source from an HP 1100 series quadrupole mass analyser (MS) (Agilent). OFA separation was carried out with a Luna C18 column (5 μm, 250 × 4.6 mm ID, Phenomenex, Torrance, CA, USA). Mobile phases were prepared by mixing ACN (A) and water (B) in gradient mode. The gradient elution was performed as follows: from 0 to 50 min the A percentage was increased from 60% to 100%; a isocratic elution at 100% A was carried out from 50 to 70 min; 1 more min was used to decrease A percentage from 100% to 60%; then, 60% A was maintained 14 more min to equilibrate column. UV–Vis detection was performed at 255 ± 10 nm (reference 500 ± 50 nm). In all cases, 20 μL were injected at a flow rate of 1 mL min^{-1}. The MS working conditions were: nebulizer gas pressure, 50 psi; drying gas flow, 9 L min^{-1} at 350 °C; vaporiser temperature, 300 °C; capillary voltage, 3 kV; corona current, 4 μA and fragmentor voltage, 60 V. The mass spectrometer was scanned within the *m*/*z* 300–500 range in the positive-ion mode.

OFAs were determined according to Rovellini and Cortesi (2004) after transesterification with 1.0 M sodium benzyloxide in benzyl alcohol. Tricaproin and triheptadecanoin were used as internal standards (results were reported in percentages as g of total OFA expressed as benzyl heptadecanoate per 100 g of oil, while benzyl caproate was used as a control for the reaction).

2.3. FTIR analysis

All spectra were acquired using a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid™ interferometer and a DigiTect™ detector system coupled to an attenuated total reflectance (ATR) accessory. The ATR accessory (Specac Inc., Woodstock, GA, USA) was equipped with a ZnSe 11 reflection crystal. All analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 (Bruker Optics) software.

For each sample (1–1.5 mL uniformly spread throughout the crystal surface), the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Three spectra were recorded for each sample. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

2.4. Data treatment and construction of data matrices

FTIR spectra were divided in 25 wavelength regions as described in Table 1. Each spectral region corresponds to a peak or a shoulder, representing structural or functional group information, either about lipids or minor components of the oil samples (see Table 1). For each region, the peak or shoulder area was measured. In order to reduce the variability associated with the total amount of oil sample used, and to minimise other sources of variance affecting the intensity of peaks, such as radiation source intensity, variables were normalised as follows: the area of each region was divided by each one of the areas of the other 24 regions; in this way, and since any pair of areas should be considered only once, the $(25 \times 24)/2 = 300$ normalised variables to be used as predictors were obtained.

For MLR studies, calibration and external validation sets were constructed. The calibration matrix contained 60 objects (which were randomly selected), which corresponded to the average of the three spectra recorded for each sample and 300 normalised variables. The external validation matrix was constructed with the remaining 12 samples also corresponding to the average of three spectra, in addition to 300 normalised variables. A response column, containing the OFA concentration (obtained by HPLC), was then added to these matrices. Statistical analyses were performed using SPSS (v. 11.5, Statistical Package for the Social Sciences, Chicago, IL, USA).

3. Results and discussion

3.1. OFA content in VOO samples

The HPLC traces in Fig. 1 (A, B and C) show that the differences in OFA content (low, medium and high) of three VOO samples are related to storage time (2 weeks, 16 months and 34 months after oil production, which corresponded to parts A, B and C, respectively). Based on the study of MS spectra, three groups of OFAs were identified (see Fig. 1): (1) isomeric forms of keto-linolenic acid (*m*/*z* 383); (2) isomeric forms of keto-linoleic acid (*m*/*z* 385); and (3) isomeric forms of keto-oleic acid (*m*/*z* 387). All *m*/*z* values corresponded to the $[M+H]^+$ ions (Rovellini & Cortesi, 2004; Rovellini et al., 1998).

The OFA content was evaluated for the 72 VOO samples, and was found to have a wide range (min = 0.3%, max = 6.5%). This can be attributed to the fact that the oil samples came from different harvest seasons and were analysed at times ranging from 1 week to 36 months after production. Rovellini and Cortesi (2004) analysed several VOOs and found that OFA percentages from 2% to 4% are typical for extra VOOs stored from 2 to 18 months at room temperature, while oil samples characterised by a total OFA higher than 4% must be considered as "expired".

By grouping the data into four groups (Fig. 2) on the basis of the OFA values, (OFA < 1.0%, 1.0% ⩽ OFA < 2.5%, 2.5% ⩽ OFA < 4% and OFA ⩾ 4%), the 72 VOOs were subdivided as follows: a first group (G1, *n* = 23) with a mean of 0.6%; a second group (G2, *n* = 15) with a mean of 1.8%; a third group (G3, *n* = 23) with a mean of 3.0% and a fourth group (G4, *n* = 11) with a mean of 5.3%. All the samples produced within one month before analysis belonged to G1 and exhibited very narrow range of OFA values (from 0.3% to 0.8%). In contrast, group G4 showed higher percentages and a wider range of variability (from 4.2% to 6.5%). These data confirm that it is pos-

Table 1
FTIR spectral regions selected as predictor variables for statistical data treatment.

Identification No.	Range, cm^{-1}	Functional group	Nominal frequency	Mode of vibration
1	3029–2989	=C–H (trans)	3025[a]	Stretching
		=C–H (cis)	3006[a]	Stretching
2	2989–2946	–C–H (CH_3)	2953[a]	Stretching (asym)
3	2946–2881	–C–H (CH_2)	2924[a]	Stretching (asym)
4	2881–2782	–C–H (CH_2)	2853[a]	Stretching (sym)
5	1795–1677	–C=O (ester)	1746[a]	Stretching
		–C=O (acid)	1711[a]	Stretching
6	1486–1446	–C–H (CH_2)	1465[b]	Bending (scissoring)
7	1446–1425	–C–H (CH_3)	1450[b]	Bending (asym)
8	1425–1409	=C–H (cis)	1417[a]	Bending (rocking)
9	1409–1396	=C–H	1400[b]	Bending
10	1396–1382	=C–H	–[b]	Bending
11	1382–1371	–C–H (CH_3)	1377[a]	Bending (sym)
12	1371–1330	O–H	1359[b]	Bending (in plane)
13	1330–1290	Non-assigned	1319[a]	Bending
14	1290–1211	–C–O	1238[a]	Stretching
		$–CH_2–$		Bending
15	1211–1147	–C–O	1163[a]	Stretching
		$–CH_2–$		Bending
16	1147–1128	–C–O	1138[b]	Stretching
17	1128–1106	–C–O	1118[a]	Stretching
18	1106–1072	–C–O	1097[a]	Stretching
19	1072–1043	–C–O	–[b]	Stretching
20	1043–1006	–C–O	1033[a]	Stretching
21	1006–929	–HC=CH– (trans)	968[a]	Bending (out of plane)
22	929–885	–HC=CH– (cis)?	914[a]	Bending (out of plane)
23	885–802	$=CH_2$	850[b]	Wagging
24	802–754	–C–H	–[b]	Bending (out of plane)
25	754–701	$–(CH_2)_n–$	723[a]	Rocking
		–HC=CH– (cis)		Bending (out of plane)

[a] According to Guillén and Cabo (1998).
[b] According to Silverstein, Bassler, and Morrill (1981).

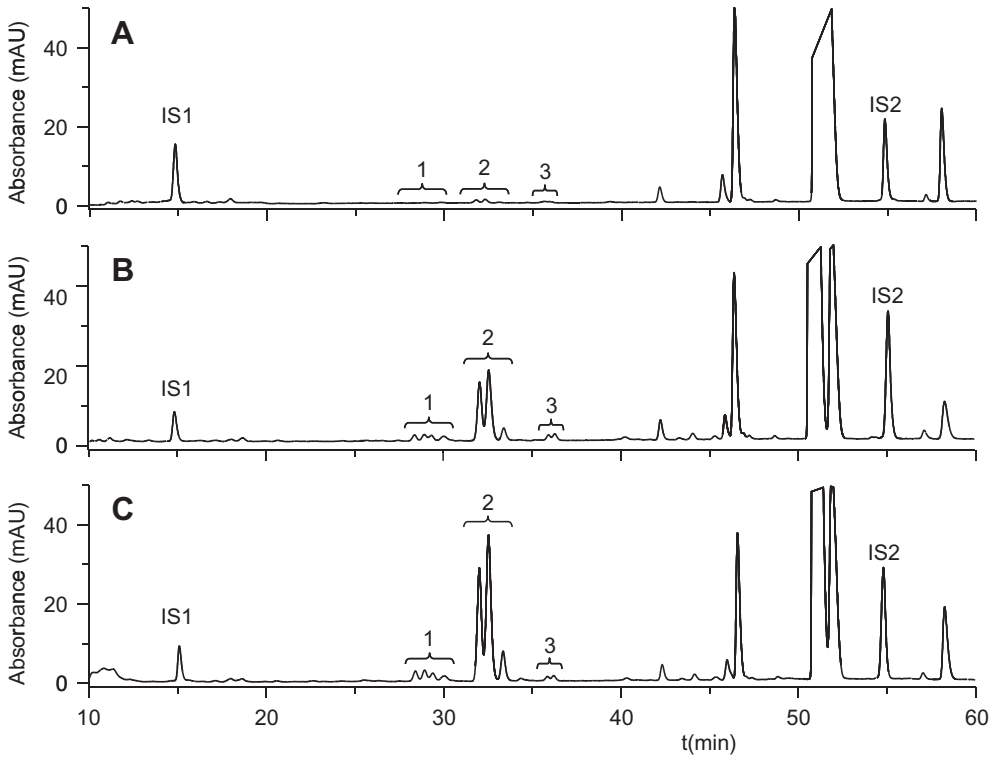

Fig. 1. OFA HPLC traces of VOOs with (A) 2 weeks, (B) 16 months and (C) 34 months after oil production. Detection was performed at 255 nm. Peak identification (as benzyl-ester derivatives): (1) group of isomeric forms of keto-linolenic acid; (2) group of isomeric forms of keto-linoleic acid; and (3) group of isomeric forms of keto-oleic acid. IS1 and IS2 (internal standards) are benzyl caproate and benzyl heptadecanoate, respectively.

sible to evaluate the freshness of VOOs with a simple OFA assay, which could also be used as an index of oxidative changes not only for olive oils, but also for other vegetable oils and fats. With OFA determination the number of analyses was reduced (i.e. peroxide

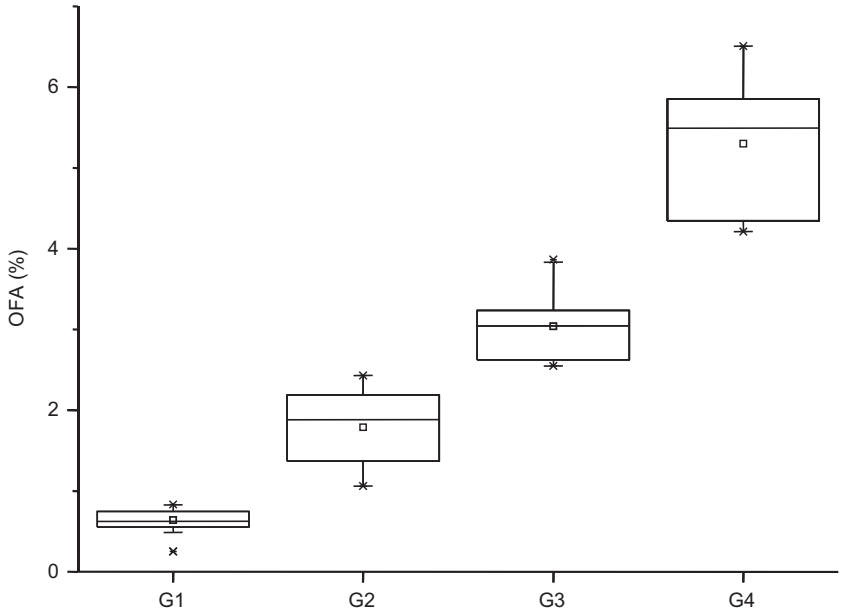

Fig. 2. Box and whisker plot showing the distribution of OFA percentages carried out by HPLC analysis in the 72 VOO samples.

values or k_{232} for primary oxidation products and *p*-anisidine value or volatile content for secondary oxidation products).

3.2. Construction of MLR models

FTIR spectra of the same three VOO samples presented in Fig. 1, characterised by different storage times and thus different OFA content, are shown in Fig. 3. As already indicated, FTIR spectra were divided into 25 wavelength regions as described in Table 1. The areas corresponding to each region were measured, normalised and used as predictors to construct MLR models. Due the elevated number of variables obtained after normalisation (300 normalised variables), the SPSS stepwise algorithm was used to select the variables to be included in the MLR models. For this purpose, the default probability values of F_{in} and F_{out}, 0.05 and 0.10 were adopted. Using the calibration matrix, two MLR models were constructed, both with and without the inclusion of an independent term (constant). The model including the constant gave lower linearity than the model without the constant (regression coefficient, *r*, of 0.944). For this reason, further studies were performed without the inclusion of the constant. The correlation plot of the predicted versus the experimental OFA percentages obtained with-

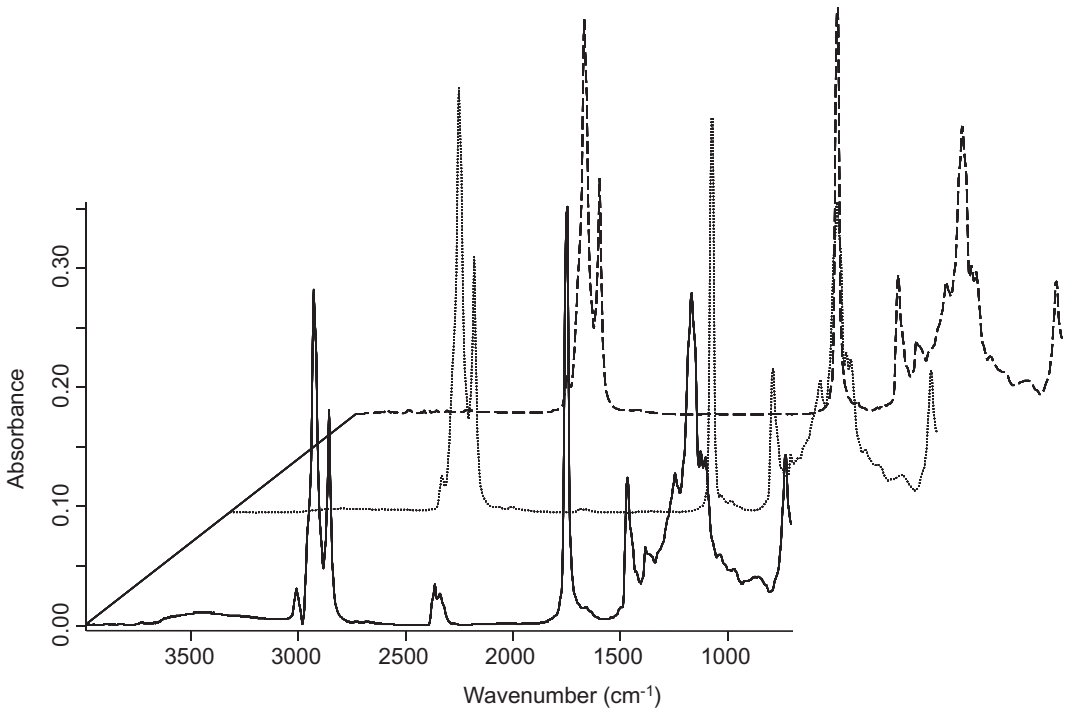

Fig. 3. FTIR spectra of VOO with 2 weeks (—), 16 months (· · ·) and 34 months (- - -) after oil production.

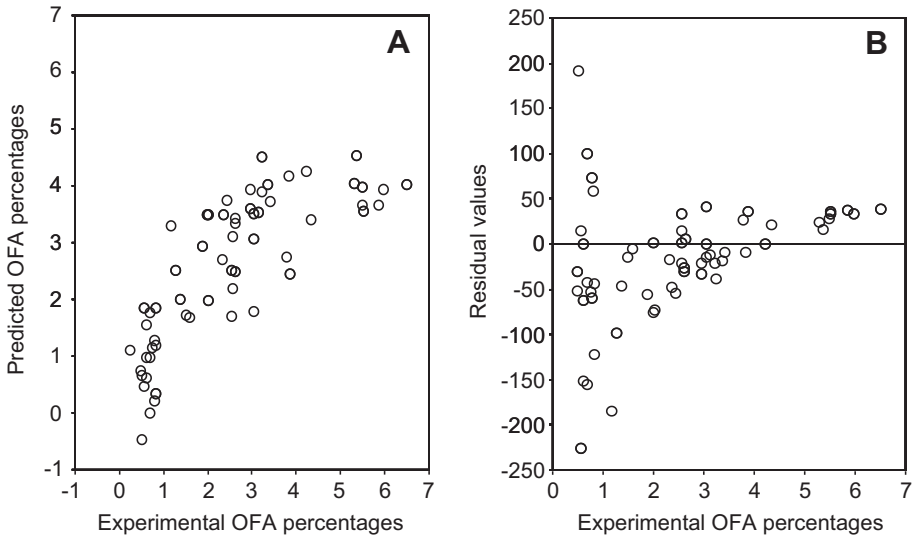

Fig. 4. (A) Correlation plot of the predicted versus the experimental OFA percentages. (B) Plot of the residual values versus the experimental OFA percentages.

out the inclusion of the constant is shown in Fig. 4, part A. When leave-one-out validation was applied, the average prediction error (calculated as the sum of the absolute differences between expected and predicted OFA percentages divided by the number of predictions) was 52%. In order to obtain information regarding the fit of the model, residual values and/or the relative errors were examined. For this purpose, a plot representing the residual values against the experimental OFA percentages (Fig. 4, part B) was obtained. A dependence of the residuals with the experimental values was observed and therefore, heteroscedasticity (non-constant variance) of the data. The problem of heteroscedasticity can be solved by either transformation of variables or by using a weighted least-square procedure (Vandeginste et al., 1998). For this reason, the following variable transformations were applied to the experimental OFA percentages: natural logarithm, square and cube roots. Homoscedasticity in the data distribution was obtained when the cube root transformation was used (see Fig. 5, part B). Comparing this plot with that in Fig. 4, part B, a homoscedasticity of the data was observed. Using cube root transformation, an r of 0.996 was obtained. The correlation plot of the predicted versus the experimental OFA percentages obtained using the cube root transformation is shown in Fig. 5, part A. The predictors selected for this model and their corresponding non-standardised coefficients (indicated between parenthesis) were the ratios of regions 3/17 (0.056), 3/20 (−0.020), 1/23 (−0.068), 19/22 (−0.041), 6/7 (−0.001), 14/22 (−0.001), 5/24 (0.003) and 12/17 (0.261). Then, the main wavelength regions selected to construct this MLR model corresponded to =C–H (trans and cis, stretching), –C–H (CH_2, stretching asym), O–H (bending in plane), C–O (stretching), –H–C=C–H– (cis?) and =CH_2 (wagging), due to the fact that these regions were those more affected by oxidation. In fact, most of these regions were characterised by a functional group with an oxygen or a double bond. When leave-one-out validation was applied, the average prediction error was 8%. When the model was applied

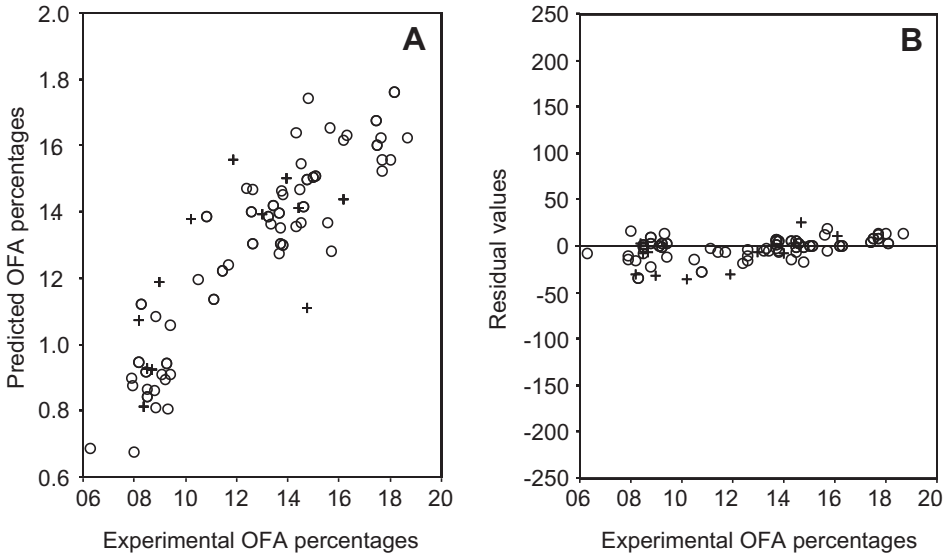

Fig. 5. (A) Correlation plot of the predicted versus the experimental OFA percentages obtained after cube root transformation. (B) Plot of the residual values versus the experimental OFA percentages obtained after cube root transformation. For both, A and B, samples were marked as calibration (○) and validation (+).

to the validation set, an excellent prediction capability was observed (see Fig. 5, part A), being the average validation error 17%.

4. Conclusions

The possibility of estimate OFA concentration in VOO by using FTIR data has been demonstrated. After a cube root transformation of the experimental OFA percentages, an MLR model constructed using eight predictors was able to predict OFA concentration with an average error of 17%.

This FTIR method is useful as an alternative of the proposed HPLC method, which main advantages included no-sample preparation and short analysis time. Thus, an extremely quick and simple procedure, which can be easily automatised, has been described.

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ARTICOLO 8

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Use of triacylglycerol profiles established by high performance liquid chromatography with ultraviolet–visible detection to predict the botanical origin of vegetable oils

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ABSTRACT

A method for the determination of triacylglycerols (TAGs) in vegetable oils from different botanical origins by HPLC with UV–vis detection has been developed. Using a core-shell particle packed column (C18, 2.6 μm), TAG separation was optimized in terms of mobile phase composition and column temperature. Using isocratic elution with acetonitrile/*n*-pentanol at 10 °C, excellent efficiency with good resolution between most of the TAG peak pairs, within a total analysis time of 15 min, was achieved. Using mass spectrometry detection, a total of 15 peaks, which were common to oils of six different botanical origins (corn, extra virgin olive, grapeseed, hazelnut, peanut and soybean) were identified. These peaks were used to construct linear discriminant analysis (LDA) models for botanical origin prediction. Ratios of the peak areas selected by pairs were used as predictors. All the oils were correctly classified with assignment probabilities higher than 95%.

1. Introduction

Vegetable oils are important commodities in the world trade and market, being widely used in several sectors of industry, including cosmetics and nutrition. Their production largely increased in the past decades, and it is still annually increasing with an estimated production of about 185 million tonnes on 2020 [1]. In particular, the production of crude vegetable oils has shown a net increase in the European Union along the last twenty years to reach about 13 million tonnes on 2008, without including olive oil, which accounted for about an additional 2 million tonnes in the 2008/2009 season [2,3].

Triacylglycerols (TAGs) are the main components of vegetable oils, as they are generally present in the 95–98% range of the whole oil composition. Their molecular structure, including the distribution of fatty acids between the different stereospecific positions of the glycerol skeleton, controls the functionality of oils and fats as food ingredients, influencing such physical properties as crystal structure and melting point. In addition, they have important physiological effects as components of the human diet, being an important source of essential fatty acids. Their imbalances can lead to several disorders such as coronary heart disease, obesity or dyslipidaemia. Thus, the development and improvement of analytical methods that enable the thorough identification of TAGs is very important to avoid adulteration of high-price quality vegetable oils with cheaper oils of lower quality and less beneficial nutritional effects.

Different chromatographic techniques are suitable for the analysis of TAG profiles in vegetable oils, but HPLC is the most employed [4]. Two HPLC techniques have been particularly used in the last years: Ag^+-HPLC in normal phase mode, based on TAG separation according to number and position of double bounds and *cis*/*trans* isomerism [5–7], and non-aqueous reversed-phase HPLC (NARP-HPLC) with mobile phase systems of low polarity, where TAG are separated according to the equivalent carbon number (ECN) [8–10]. Separation according to the different position of double bond(s) or within ECN groups is also possible [10–12]. In addition, comprehensive 2D HPLC, based on the combination of an anionic exchange column charged with silver ions followed by an RP-C18 silica monolithic column as second dimension has been recently applied for the characterization of TAGs in complex lipid matrices [13].

Several detectors are generally coupled with NARP-HPLC for TAG analysis. UV detection at low wavelengths (205 or 210 nm) provide a linear response; however, a low sensitivity for saturated TAG has been reported [10,14]. Evaporative light scattering

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is also employed, but it is not appropriate for quantitative analyses due to the non-linear response [10], while refractive index detection, although often used for routine quality control, cannot be applied with gradient elution [15]. Several applications reporting mass spectrometry (MS) detection, in combination with atmospheric pressure chemical ionization (APCI) as ionization source (which provides linear responses and excellent sensitivity with both saturated and unsaturated TAGs), have been recently published [12,16,17].

Finally, the application of multivariate statistical techniques to the TAG chromatographic data can lead to the discrimination of oils according to their botanical origin. Principal component analysis, linear discriminant analysis (LDA) and cluster hierarchical analysis have been all successfully applied for this purpose [8,17,18].

The aim of this work was the development of an analytical method based on the use of HPLC columns of the new core-shell particle class, for the prediction of the botanical origin of vegetable oils, based on the determination of the TAG profile. For this purpose, NARP-HPLC with UV–vis detection, with the aim of obtaining a good resolution within a short analysis time, was used. TAG profiles were used to construct LDA models addressed to the prediction of vegetable oil origins.

2. Experimental

2.1. Reagents and samples

The following analytical grade reagents were used: acetonitrile (ACN), 2-propanol, ethanol (EtOH), *n*-butanol, *n*-pentanol (Scharlau, Barcelona, Spain) and *n*-hexane (Riedel-de-Haën, Seelze, Germany); OOO (Sigma, Saint Louis, MO, USA) was used as standard. Deionized water (Barnstead deionizer, Sybron, Boston, MA, USA) was also used. A total of 36 vegetable oils were employed in this study, six for each botanical origin. These samples, which were either purchased in the local market or kindly donated by different manufacturers, were the following: corn oil from Guinama, Asua, Cristal, Gloria and Mazola; extra virgin olive oil (EVOO) from Borges, Carbonell, Coosur, Grupo Hojiblanca, Romanico and Torrereal; grapeseed oil from Coosur, Guinama and Paul Corcelet; hazelnut oil from Guinama and Percheron Freres; peanut oil from Coppini, Guinama and Maurel; and soybean oil from Coosur, Guinama, Biolasi and Coppini. In all cases, the botanical origin and quality grade of all the samples were guaranteed by the suppliers.

2.2. Instrumentation and working conditions

A 1100 series liquid chromatograph provided with a quaternary pump, a degasser, a thermostated column compartment, an automatic sampler and an UV–vis diode array detector (Agilent Technologies, Waldbronn, Germany) was used. Separation was carried out with a Kinetex™ C18 100A column (150 mm × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). The optimized separation conditions were: isocratic elution with a 70:30 ACN/*n*-pentanol mixture; UV–vis detection at 205 ± 10 nm (360 ± 60 nm as reference); column temperature, 10 °C; flow rate, 1.5 mL min^{-1} and injection volume, 20 μL.

When required, the liquid chromatograph was also coupled (in series with the UV–vis detector) to the APCI source of an HP 1100 series quadrupole mass spectrometer (MS) (Agilent). The MS working conditions were as follows: nebulizer gas pressure, 35 psi; drying gas flow, 12 L min^{-1} at 350 °C; vaporizer temperature, 350 °C; capillary voltage, 3 kV. Nitrogen was used as nebulizer and drying gas. The mass spectrometer was scanned within the *m*/*z* 300–1000 range in the positive-ion mode.

2.3. Sample preparation, data treatment and statistical analysis

Vegetable oil samples were chromatographed after a simple dilution to 3% in a 2:2:1 ACN/2-propanol/*n*-hexane (v/v/v) ternary mixture. All samples were injected three times. A data matrix was constructed measuring the APCI-MS peak areas of all peaks, and using these data as original variables. After normalization of the variables, statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL, USA). LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained [19]. This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of λ_w approaching zero are obtained with well resolved categories, whereas overlapped categories made λ_w to approach one. Up to $N-1$ discriminant vectors are constructed by LDA, being N the lowest value for either the number of predictors or the number of categories. The selection of the predictors to be included in the LDA models was performed using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or *F*-test. However, the entrance of a new predictor modifies the significance of those predictors which are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The probability values of F_{in} and F_{out}, 0.01 and 0.10, respectively, were adopted.

3. Results and discussion

3.1. Optimization of the separation of TAGs

In order to obtain TAG separation with optimal resolution within a short analysis time, the use of a Kinetex™ core-shell particle column was tried. These columns are capable of maintaining high efficiencies at increasing flow rates with the subsequent reduction of analysis time. Also, these columns operate comfortably within the pressure limits of conventional LC instruments, rivalizing the performance obtained with sub-2 μm particle columns on UHPLC instruments.

Peanut oil was used to optimize TAG separation in terms of mobile phase composition and column temperature. A flow rate of 1.5 mL min^{-1}, which gave a moderate pressure, was selected. The influence of the length of the alkyl chain of different alcohols used to modify eluotropic strength was also investigated. For this purpose, a column temperature of 25 °C and binary mixtures of ACN with either EtOH, 2-propanol, *n*-butanol or *n*-pentanol, all at a 70:30 ratio, were tried. Using EtOH, poor resolution and long analysis times (*ca.* 90 min) were obtained (data not shown). Using 2-propanol (Fig. 1A), resolution largely improved, being analysis time shorter than with mobile phases containing EtOH. Analysis time further decreased using *n*-butanol and *n*-pentanol (Fig. 1B and C), being also resolution slightly improved. Pump pressures ranged from 16.7 to 20.0 MPa for mobile phases containing EtOH and *n*-pentanol, respectively. Thus, an ACN/*n*-pentanol mixture was selected for the following studies. Next, proportions of ACN/*n*-pentanol from 80:20 to 50:50 were tried in isocratic elution mode. With 50:50 ACN/*n*-pentanol, most peaks overlapped. Resolution slightly improved when ACN content was increased to 60%, and further increased with 70% ACN; however, resolution decreased

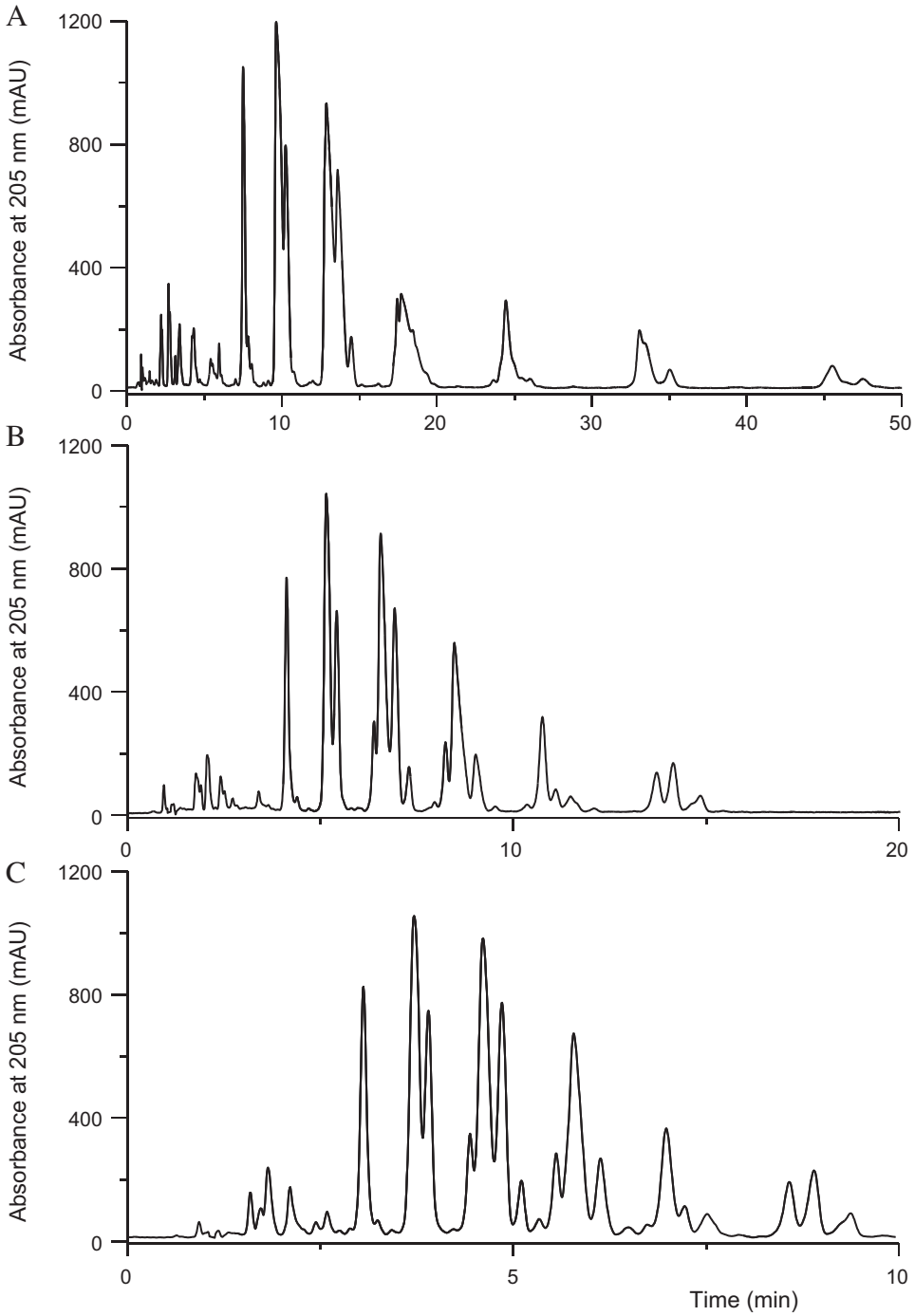

Fig. 1. Influence of the length of the alkyl chain of the alcohol modifier on the TAG separation by isocratic elution with 70:30 ACN/alcohol using: 2-propanol (A), *n*-butanol (B) and *n*-pentanol (C). Chromatographic conditions: column temperature, 25 °C; flow rate, 1.5 mL min^{-1}.

and analysis time increased (up to 40 min) when an 80:20 ACN/*n*-pentanol mixture was used. Thus, 70:30 ACN/*n*-pentanol was selected. Finally, column temperature was varied between 6 and 25 °C. Resolution increased when temperature decreased, although both pump pressure and analysis time also increased. Thus, a temperature of 10 °C, which gave 22.0 MPa (Fig. 2), was selected.

3.2. Characterization of the TAG profiles of vegetable oils and TAG peak identification

The optimized separation method was applied to the analysis of vegetable oil samples. Chromatograms of a corn (A), EVOO (B), grapeseed (C), hazelnut (D), peanut (E) and soybean (F) oils are shown in Fig. 2. A total of 26 peaks were identified in less than 15-min chromatograms. Peak identification in oil samples of all categories was performed using MS detection (peak assignments are given in Table 1). A series of standard solutions containing decreasing concentrations of OOO were used to estimate the limit of detection (LOD) of the TAGs. According to the 3*s* criterion (which estimates the background noise with the solution of lower concentration, and which considers as LOD any signal with at least a response higher than 3 times the standard deviation of background noise), a LOD of 1 μg mL^{-1} was estimated being repeatability of peak areas 3% (n = 8). Considering that vegetable oil

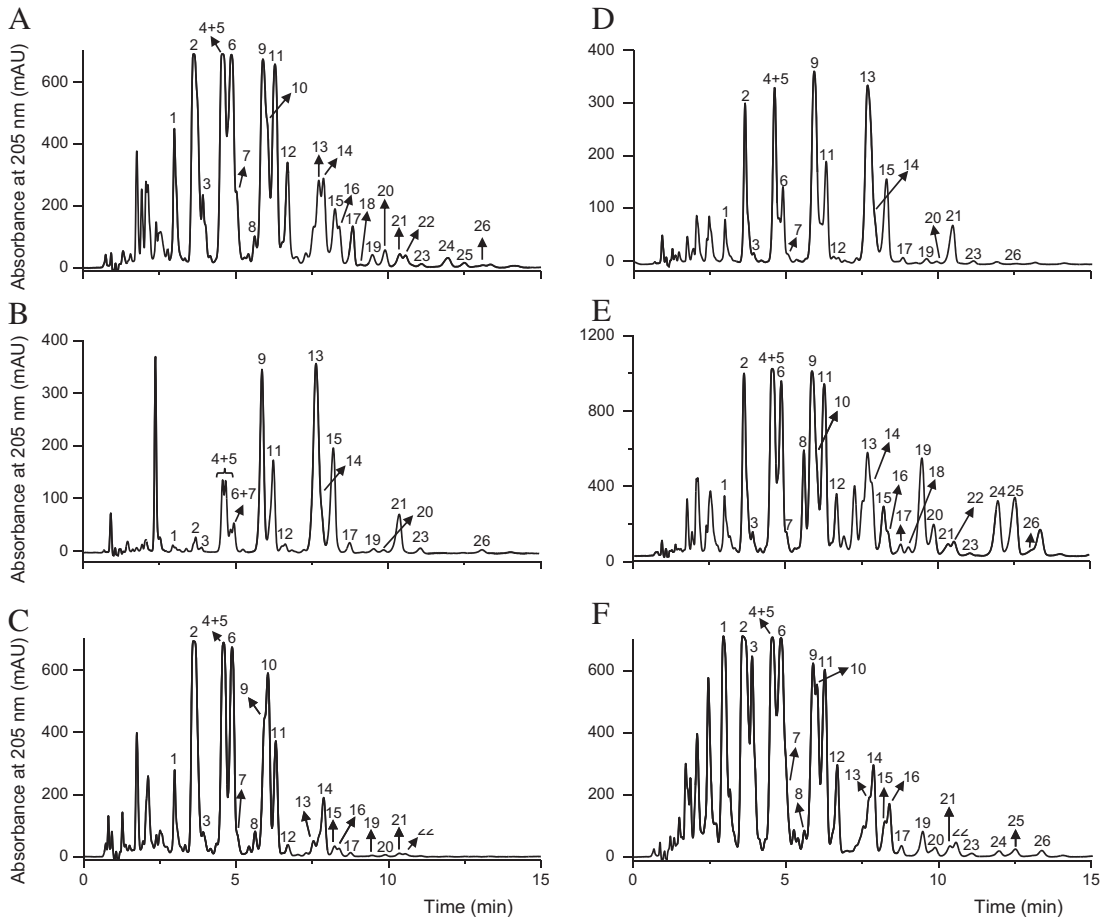

Fig. 2. Chromatograms showing the TAGs profiles of a corn (A), EVOO (B), grapeseed (C), hazelnut (D), peanut (E) and soybean (F) oil samples. Chromatographic conditions: isocratic elution with 70:30 ACN/*n*-pentanol; column temperature, 10 °C. Other conditions as in Fig. 1. Peak identification according to Table 1.

Table 1
TAGs identified by APCI-HPLC–MS analysis of vegetable oils.

Peak no.[a]	TAG[b]	PN	$[M+H]^+$	$[M+H-R_1COOH]^+$	$[M+H-R_2COOH]^+$	$[M+H-R_3COOH]^+$
1	LLLn	40	877.7	LL 599.5	LLn 597.5	–
2	LLL	42	879.7	LL 599.5	–	–
3	OLLn	42	879.7	LLn 597.5	OLn 599.5	OL 601.5
4	OLL	44	881.8	OL 601.5	LL 599.5	–
5	OLPo	44	855.7	OL 601.5	LPo 573.5	OPo 575.5
6	LLP	44	855.7	LP 575.5	LL 599.5	–
7	OLnO	44	881.8	OLn 599.5	OO 603.5	–
8	GLL	46	909.8	LL 599.5	GL 629.5	–
9	OLO	46	883.8	OL 601.5	OO 603.5	–
10	SLL	46	883.8	SL 603.5	LL 599.5	–
11	OLP	46	857.8	OL 601.5	LP 575.5	OP 577.5
12	PLP	46	831.7	PP 551.5	LP 575.5	–
13	OOO	48	885.8	OO 603.5	–	–
14	SLO	48	885.8	OL 601.5	SL 603.5	SO 605.5
15	OOP	48	859.8	OO 603.5	OP 577.5	–
16	PSL	48	859.8	SL 603.5	PS 579.5	LP 575.5
17	PPO	48	833.8	OP 577.5	PP 551.5	–
18	LLBe	50	939.8	LBe 659.6	LL 599.5	–
19	PPP	48	807.7	PP 551.5	–	–
20	POG	49	887.8	OP 577.5	PG 605.5	GO 631.6
21	SOO	50	887.8	OO 603.5	SO 605.5	–
22	SSL	50	887.8	SL 603.5	SS 607.6	–
23	SOP	50	861.8	OP 577.5	PS 579.5	SO 605.5
24	OLBe	51	941.8	OL 601.5	LBe 659.6	OBe 660.6
25	OOA	52	915.8	OO 603.5	OA 633.6	–
26	SSO	52	889.8	SO 605.5	SS 606.6	–

[a] Peak identification number according to Fig. 2; TAGs identified according to the protonated molecule ($[M+H]^+$) and diacylglycerol ions observed in the APCI mass spectrum, and to the relative order of partition numbers (PN).

[b] Structure indicated by fatty acid composition (e.g. OOO for triolein) using the following abbreviations: Po, palmitoleic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Ln, linolenic acid; A, arachidic acid; G, gadoleic acid; Be, behenic acid.

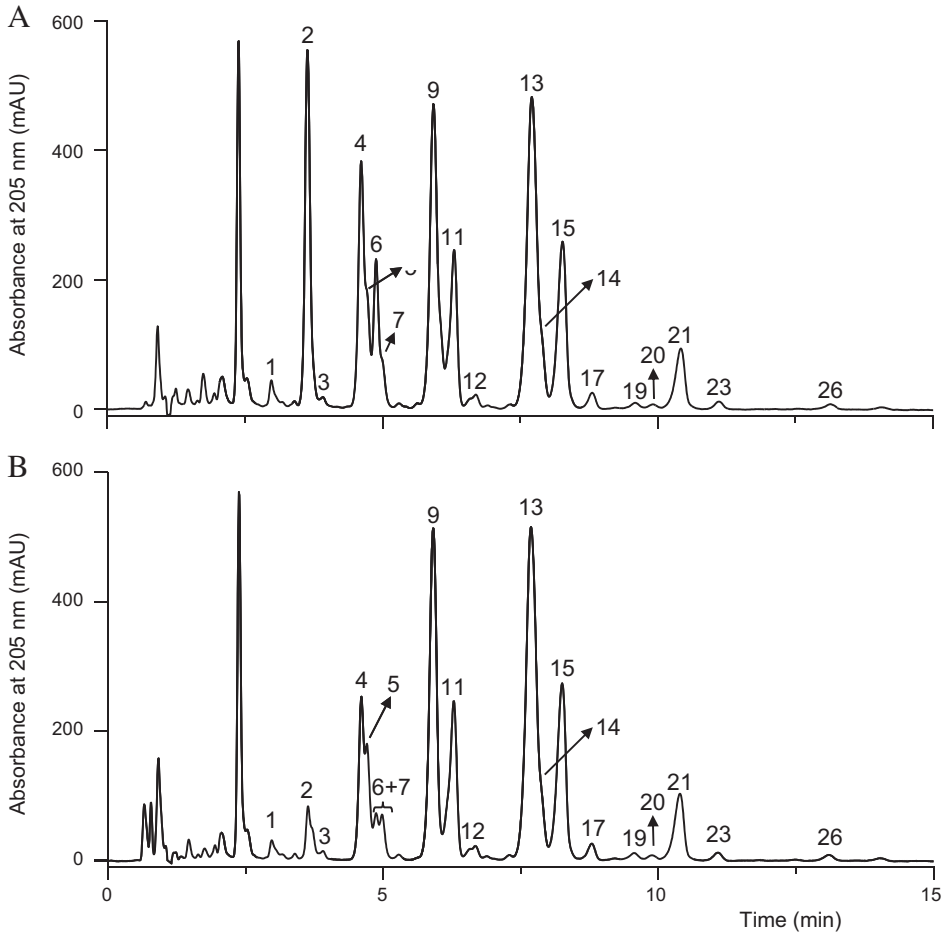

Fig. 3. Chromatograms showing the TAG profiles of binary mixtures containing 90% EVOO and 10% of either grapeseed (A) or hazelnut (B) oils. Other conditions as in Fig. 2.

samples were diluted to 3% in a 2:2:1 ACN/2-propanol/*n*-hexane (v/v/v) ternary mixture, the obtained LOD (1 $\mu g\,mL^{-1}$) correspond to a 33 $\mu g\,g^{-1}$ of oil samples. The percentages of TAGs found in the total TAG fraction of the vegetable oil samples analysed, calculated from the APCI-MS peak areas, are given in Table 2; in all cases, RSD values below 8% were obtained. The great differences between the TAG percentages for oils with a different origin (Fig. 2) made these values to be useful as discriminant variables.

3.3. Identification of EVOO adulteration with other low cost oils

In order to evaluate the possibility of detecting EVOO adulteration with low cost oils, binary mixtures containing a 90% EVOO with a 10% of either corn, grapeseed, hazelnut, peanut or soybean oils were prepared. When EVOO was adulterated with grapeseed oil, the relative areas of peaks 4 and 6 (corresponding to OLL and LLP, respectively) and particularly that of peak 2 (corresponding to LLL) largely increased (Fig. 3A). Similarly, the presence of hazelnut oil was evidenced by an increase of the areas of peaks 2 and 4 (LLL and OLL, respectively), the areas of peaks 9 and 13 (OLO and OOO, respectively) also increasing slightly (Fig. 3B). The adulteration with corn oil gave a large increase of the areas of peaks 2, 4 and 6 (LLL, OLL and LLP, respectively), as well as a moderate increase of the areas of peaks 1, 3, 9 and 12 (LLLn, OLLn, OLO and PLP, respectively). When peanut oil was present, the areas of peaks 2, 4, 6 and 18 (LLL, OLL, LLP and LLBe, respec-

Table 2
Mean percentages of the TAGs found in the total TAG fraction of vegetable oils of different botanical origin.

TAG	Corn	EVOO	Grapeseed	Hazelnut	Peanut	Soybean
LLLn	5.0	1.9	4.7	2.8	3.3	10.4
LLL	14.2	2.0	21.2	9.8	8.9	14.9
OLLn	2.9	0.9	1.9	0.8	0.9	7.3
OLL + OLPo	13.3	9.6	18.7	12.7	11.8	12.3
LLP	12.0	1.5	14.4	5.3	8.9	11.2
OLnO	2.5	2.4	1.2	–	1.1	2.2
GLL	1.1	–	1.6	–	4.8	1.0
OLO	10.4	17.6	8.8	20.0	11.5	7.5
SLL	4.9	–	10.1	–	2.7	5.8
OLP	10.5	9.9	6.6	8.1	8.8	8.0
PLP	4.9	1.8	1.1	0.9	3.2	3.7
OOO	3.8	24.7	1.2	20.7	7.2	2.0
SLO	3.5	3.2	4.9	3.4	2.1	3.4
OOP	2.7	12.7	0.9	8.0	2.9	1.3
PSL	1.7	–	0.7	–	0.7	2.2
PPO	1.8	1.7	0.5	0.9	0.7	0.6
LLBe	0.2	–	–	–	0.6	–
PPP	0.7	1.0	0.4	0.6	6.5	1.4
POG	0.9	0.9	0.4	0.5	2.0	0.7
SOO	0.7	5.8	0.5	4.3	0.9	0.6
SSL	0.7	–	0.4	0.5	0.9	0.8
SOP	0.3	1.3	–	–	0.4	0.5
OLBe	0.8	–	–	–	4.1	0.7
OOA	0.3	–	–	–	4.6	0.8
SSO	0.2	1.1	–	0.8	0.5	0.8

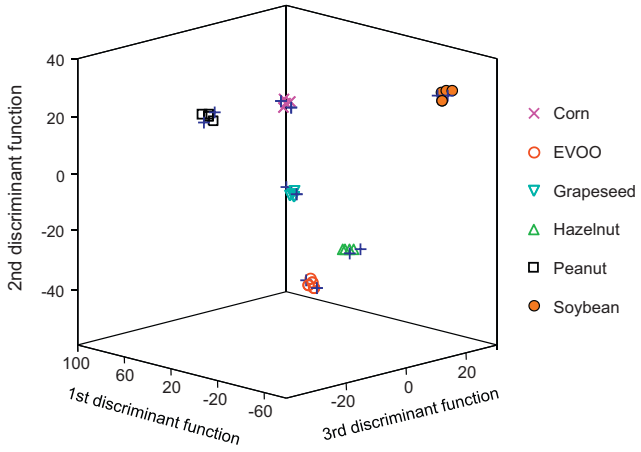

Fig. 4. Score plot on an oblique plane of the 3D space defined by the three first discriminant functions of the LDA model constructed to classify vegetable oils according to their botanical origin. Evaluation set samples indicated with crosses.

tively) increased largely, and the areas of peaks 1, 9, 11, 12 and 20 (LLLn, OLO, OLP, PLP and POG, respectively) increased slightly, while other peaks, not present in EVOO, were also now evidenced (peaks 8, 24 and 25 which corresponded to GLL, OLBe and OOA, respectively). Finally, adulteration with soybean oil produced a large increase of the areas of peaks 1, 2, 3, 4 and 6 (LLLn, LLL, OLLn, OLL and LLP, respectively). Therefore, the adulteration of EVOO with small percentages of other oils was clearly evidenced in all cases, although with a moderate sensitivity for hazelnut oil.

3.4. Normalization of the variables and construction of LDA models

In order to construct classification models, only the TAGs which were observed to be common to the oils of all the botanical origins were used as predictors. This corresponded to a total of 15 peaks (since the peaks of OLL and OLPo overlapped). To reduce the variability associated to sources of variance that can affect the sum of the areas of all the peaks, normalized rather than absolute peak areas were used. In order to normalize the variables, the area of each peak was divided by each one of the areas of the other 14 peaks; in this way, and taking into account that each pair of peaks should be considered only once, $(15 \times 14)/2 = 105$ non-redundant peak area ratios were obtained to be used as predictors. Using the normalized variables, an LDA model capable of classifying the vegetable oil samples according to their respective botanical origin was constructed. A matrix containing 36 objects (6 origins × 6 samples of each origin) and 105 predictors was constructed. A response column, containing the categories corresponding to the 6 botanical origins, was added. This matrix was divided in two groups of objects to constitute the training and evaluation sets. The training set was composed by 6 groups of 4 samples (a group for each botanical origin, $6 \times 4 = 24$ objects), while the evaluation set was constituted by the remaining samples (12 objects). When the LDA model was constructed, an excellent resolution between all the category pairs was achieved (Fig. 4, $\lambda_w = 0.01$). The variables selected by the SPSS stepwise algorithm, and the corresponding standardized coefficients of the model, showing the predictors with large discriminant capabilities, are given in Table 3. According to this table, the main peak area ratios selected by the algorithm to construct the LDA model corresponded to the following TAG concentration ratios: (OLL + OLPo)/OOO, OLP/PLP, LLL/OOO, OLLn/SLO and (OLL + OLPo)/OOO. Using this model and leave-one-out validation, all the objects of the training set were correctly classified. Concerning to the prediction capability of the model, all the objects of the evaluation set (represented with crosses in Fig. 4) were correctly assigned within a 95% probability level. In addition to this, when a mixture of oils is evaluated by the constructed LDA model, this mixture was assigned to a category with a lower probability of assignation, which indicate that the studied oil can be a mixture of oils.

Table 3
Predictors selected and corresponding standardized coefficients of the LDA model constructed to classify vegetable oils according to their botanical origin.

Predictors[a]	f_1	f_2	f_3	f_4	f_5
LLL/(OLL + OLPo)	−0.86	1.53	−1.97	−0.49	0.72
LLL/OOO	−10.4	0.52	1.95	−1.06	−0.06
OLLn/SLO	9.35	−6.47	0.10	2.75	−0.71
(OLL + OLPo)/OLP	−1.74	5.64	2.19	0.36	−1.11
(OLL + OLPo)/PLP	5.97	−13.6	−11.7	3.15	4.22
(OLL + OLPo)/OOO	13.1	4.72	1.83	10.2	4.24
LLP/SOO	3.40	0.17	0.22	2.85	−1.28
OLO/PLP	2.22	−3.93	5.17	−1.09	1.55
OLO/SOO	−1.60	−2.40	−2.12	−2.70	0.04
OLP/PLP	−13.0	23.0	7.56	−6.51	−4.28
OOO/SOO	0.42	9.38	8.90	4.96	4.70

[a] Ratios of TAG peak areas.

4. Conclusions

The feasibility of classifying vegetable oils according to their botanical origin by using TAG profiles obtained by HPLC-UV–vis followed by LDA of the chromatographic data has been demonstrated. Using conventional normal-pressure HPLC with a core-shell particle packed column, a short analysis time, while preserving high efficiency and large resolution between most TAG peak pairs, was achieved. Thus, due to speed and simplicity, the proposed method is of interest for the routine quality control of quality vegetable oils. This method can be conveniently applied to oil screening to detect adulteration.

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ARTICOLO 9

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ORIGINAL ARTICLE

Application of Differential Scanning Calorimetry-Chemometric Coupled Procedure to the Evaluation of Thermo-Oxidation on Extra Virgin Olive Oil

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Abstract In this work, the opportunity of adopting a differential scanning calorimetry (DSC)-principal component analysis (PCA) coupled procedure to measure the degree of thermal stress for extra virgin olive oil (EVOO) was presented. Oil was subjected to thermal stress under convectional or microwave heating treatments at different heating times up to 1,440 and 15 min, respectively, and *p*-anisidine values (PAV) were obtained on all samples to measure their oxidative degradation. The entire DSC profiles obtained on the oil upon cooling in the range from 30 °C to –80 °C and subsequent re-heating to 30 °C at different times and under cooking procedures have been subjected to PCA data analysis. PCA discriminated samples by means of profile changes in DSC transition both upon cooling and heating not only according to treatment times (which accounted for the degree of thermo-oxidation) but also considering different heating process. The proposed procedure may be useful to measure oil thermal stress and to select appropriate heating condition to be applied for EVOO both in industrial and/or in food-catering sectors.

Keywords Differential scanning calorimetry · Extra virgin olive oil · Thermo-oxidation · Chemometric procedure

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Introduction

Extra virgin olive oil (EVOO) is a well known vegetable oil, which takes a consistent part of the Mediterranean diet for its health promoting effects related to the prevention of oxidative damage, as it was recently stated by the European Food Safety Authority (EFSA) panel on Dietetic Products, Nutrition and Allergies in relation to its polyphenol content.[1] Its balanced chemical composition characterized by high content of monounsaturated (oleic acid in particular) fatty acids, a proportioned presence of polyunsaturated ones and such minor components as phenolic compounds, tocopherols, and carotenoids, known to act as antioxidants against reactive species, can explain its relatively high stability against oxidative sequence involving lipid molecules due to storage and/or thermal treatments.[2–4]

Differential scanning calorimetry (DSC) is a thermoanalytical technique widely employed for the evaluation of quality parameters of vegetable oil as thermal properties obtained by cooling and heating transitions were related to chemical composition.[5] Recently, correlations among thermal properties and both major and minor chemical components were also established for EVOO.[6] Its use presents several advantages as it does not require time-consuming

manipulation practices and chemical treatment of the sample, avoiding the use of toxic chemicals that could be hazardous for the analyst and the environment, with well-automated analysis protocol that papers recently published have estimated to be very helpful also for the evaluation of EVOO adulteration.[7,8]

Among DSC applications in the field, its use for assessing oxidative deterioration of vegetable oils is well known and reviewed.[9,10] Several aspects were taken into consideration: the evaluation of oil stability by extrapolating induction time from isothermal curve, which were found to be related to those measured by common tests as OSI[11] or Rancimat,[12] the monitoring of the immediate quality and the expected oxidation stage after such thermal treatments as deep-frying[13,14] or microwave heating[15,16], as changes in DSC thermal properties were found to be strictly correlated to those of standard oxidative stability indices (peroxide and anisidine values, free fatty acids, etc.).

Changes in DSC curves and related thermal properties were also discussed for olive oils after conventional[17] and microwave[18,19] thermal oxidation carried out under different conditions (i.e. temperature, time, microwave power).

In all these previous works, DSC thermograms obtained as a result of oil composition and their processing were treated by evaluating cooling and heating transition profiles and by extracting such thermal properties as enthalpy, onset, offset and peak temperatures of transitions from the curves. The chemometric processing of digitized calorimetric curves is also possible, being an alternative and attractive approach to such treatment of DSC data. Nevertheless, few examples are reported in literature, especially in the area of food research.[20–22] In a previous work, a novel strategy based on coupling DSC to partial least square (PLS) chemometric methodology for the determination of fatty acid composition of olive oil was developed, presenting a suitable model for the determination of oleic acid content overall, which is related to oil health benefits.[23]

Multivariate methods such as principal component analysis (PCA) is useful to visualize representative features in multidimensional data, by reducing noise and data dimension. Given data matrix $X_{(p\times t)}$, the PCA algorithm performs a linear transformation of the set of random vectors x_i $(i=1\ldots p)$ into a new set of vectors (w_i, where $i=1\ldots p$), the principal components (PC's). These PC's are uncorrelated and are ordered according to their ability to explain variation of the data, so that the first few retain most of the variation present in the original variables. The first PC is oriented in the direction on which the variance of the projection of the original vector is maximized, while each of the subsequent PC's is defined in the same way, being orthogonal to all the previous PC's. The loadings matrix contains a column-wise arrangement of the weights (contribution) of the original variables on PCs. The score matrix is the product which contains information regarding data variation.[24]

In this work, the entire DSC profile obtained upon cooling in the range from 30 °C to −80 °C and subsequent reheating up to 30 °C on an EVOO sample has been subjected to multivariate data analysis with the aim to analyse the main calorimetric changes that occur during different thermal treatments (convection oven, OV, and microwave, MW) and to explore the opportunity of adopting a DSC-PCA coupled procedure as an accurate measurement of thermal stress degree. Compositional data on raw sample and *p*-anisidine values (PAV) values of heated oils were also provided.

Material and Methods

Samples

EVOO sample was supplied by Coppini Arte Olearia (Parma, Italy). The olives used for oil production were hand-picked in 2009 and belonged to two cultivars (*Nocellara del Belice* and *Ogliarola Messinese*) from Trapani (Sicily, Italy); olives were processed by a continuous industrial plant with a working capacity of 1 ton h^{-1} equipped with a hammer crusher, a horizontal malaxator (at a temperature of 27 °C), and a three-phase decanter. The sample was stored in a dark bottle without headspace at room temperature (23±1 °C) before analysis.

Thermal Treatment

Thermo-oxidation conditions were chosen on the basis of earlier works taking into account related changes in DSC profiles previously observed.[17–19] For conventional heating, an electric oven (OV) with air convection was used (FVQ105XE, Electrolux Rex, Pordenone, Italy). Three aliquots (90 ml) of oil were placed in opened 150 ml flasks (7.3 cm i.d.) and exposed at a temperature of 180 °C for 0, 30, 90, 120, 180, 360, 900 and 1,440 min.

For microwave heating (MW), a domestic oven was used (Perfect Combo MW 651, DeLonghi, Treviso, Italy). Three aliquots of oil were prepared as in conventional heating, placed on the rotatory turntable plate of the oven at equal distance and exposed at a frequency of 2,450 Hz at medium power (720 W). The oil samples were heated for 0, 3, 4.5, 6, 9, 12 and 15 min.

All heated samples were allowed to cool at room temperature (23±1 °C) for 60 min after thermal treatment prior to be immediately analysed.

Chemical Analysis

Triacylglycerols (TAG) were analysed by HPLC coupled to both diode-array (DAD) and mass spectrometer (MSD) detectors, as previously described.[18] TAG were tentatively identified based on their UV–vis and mass spectra obtained by HPLC-DAD/MSD and literature data.[25] The results were expressed as normalized area in percentage (%). TAG are grouped according to the type of FA bonded to the glycerol structure as monosaturated triacylglycerols (MSTAG), disaturated triacylglycerols (DSTAG) and triunsaturated triacylglycerols (TUTAG). The following TAG were identified: trilinolein (LLL), dilinoleoyl-palmitoleoyl-glycerol (LLPo), oleoyl-linoleoyl-linolenoyl-glycerol (OLLn), dilinoleoyl-oleoyl-glycerol (OLL), palmitoleoyl-oleoyl-linoleoyl-glycerol (OLPo), dilinoleoyl-palmitoyl-glycerol (LLP), dioleoyl-linolenoyl-glycerol (OLnO), dioleoyl-linoleoyl-glycerol (OLO), palmitoyl-oleoyl-linoleoyl-glycerol (OLP), dioleoyl-palmitoleoyl-glycerol (OOPo), palmitoyl-palmitoleoyl-oleoyl-glycerol (POPo), triolein (OOO), stearoyl-oleoyl-linoleoyl-glycerol (SLO), dipalmitoyl-oleoyl-glycerol (POP), dioleoyl-stearoyl-glycerol (SOO) and palmitoyl-stearoyl-oleoyl-glycerol (SOP).

Fatty acid (FA) composition was determined according to Bendini et al.[26], as methyl esters by capillary gas chromatography (GC), equipped with a flame ionization detector (FID), after alkaline treatment. The results were expressed as normalized area in percentage (%). Fatty acids were also reported according to their unsaturation degree, as saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Evaluation of free acidity (expressed as% oleic acid) and primary oxidation products (POV, expressed as meq O_2/kg lipids) were performed according to the official methods described in annex III of EEC Regulation 2568/91.[27] PAV determination was performed according to the IUPAC standard method 2.504, by measuring absorbance at 350 nm, on fresh and thermo-oxidized samples.[28] Three replicates for each determination were analyzed per sample.

DSC Analysis

Samples of oil (8–10 mg) were weighed in aluminium pans, covers were sealed into place. Then, they were analyzed using DSC Q100 (TA Instruments, New Castle, DE, USA). Indium (melting temperature 156.6 °C, ΔH_f=28.45 Jg^{-1}) and *n*-dodecane (melting temperature −9.65 °C, ΔH_f= 216.73 Jg^{-1}) were used to calibrate the instrument and an empty pan was used as reference. Oil samples were equilibrated at 30 °C for 3 min and then cooled at −80 °C at the rate of 2 °C min^{-1}, equilibrated at −80 °C for 3 min and then heated from −80 °C to 30 °C at 2 °C min^{-1}. Dry nitrogen was purged in the DSC cell at 50 cm^3 min^{-1}. Thermograms were analyzed with Universal Analysis Software (Version 3.9A, TA Instruments) in order to be exported in an ASCII compatible format. Three replicates were analysed per sample.

Data Processing and PCA Models

Thermograms, in ASCII compatible format, were imported to Matlab (Mathworks Inc., Natick, MA, USA.) using routines written *ad hoc*. PCA was carried out employing Tomcat Toobox[29] routine written for Matlab. PCA model was computed on overall data. The data were pre-treated in order to be transformed into a suitable form for PCA. First, the signal correction was done considering the whole unfolded thermogram. Then, the data were mean-centred (MC) (Figure 3a), providing that all the samples appear to have the same mean signal level as the ideal. The replicates were averaged and the mean was taken as individual sample. All programs were run on an ACER-Aspire 5050 computer with an AMD Turion™ 64 Mobile, 2.20 GHz microprocessor and 2.00 Gb of RAM.

Results and Discussion

Chemical and DSC Analysis

Chemical composition and quality parameters of EVOO are reported in Table 1. Eight TAG were separately quantified while the others were quantified as pairs (LLL + LLPo, OLL + OLPo, LLP + OLnO and OLP + OOPo). TUTAG were higher than the other groups of TAG as OOO represents the main TAG in EVOO. FA percentages fell within the range recently indicated by the Commission Regulation (EC, 2011) with an oleic/linoleic acid (O/L) ratio higher than 7 (~10.7), which is considered as the limit value to be overcame for oil oxidative stability. Free acidity of the oil was 0.4%, being below the limit (≤0.8%) set for this commercial category by the EC Regulation.[30]

Initial oxidative status of unheated oil was measured both by POV and PAV with the former value lower than that (≤20) indicated by the European Commission Regulation[30] for this high quality oil, which is consumed crude, and the latter similar to that generally found in fresh oil.[3]

Thermo-oxidation of oil samples was evaluated only by PAV, which measure oxidation products (alkanes, alkenes, aldehydes, and ketones) formed by primary oxidation products (i.e. peroxides) mainly in final reaction stages. Data are summarized in Table 2 for all samples. PAV values increased to reach 102 after 900 min of treatment for conventionally heated oil. Values reached by samples at the longest time of treatment were not reported as it could be considered too high to be significant.

Table 1 TAG, main FA composition and quality parameters of EVOO sample

TAG (%)[a]	
LLL + LLPo	3.3
OLLn	1.3
OLL + OLPo	13.3
LLP + OLnO	4.3
OLO	21.4
OLP + OOPo	11.4
POPo	1.6
OOO	25.6
SLO	11.8
POP	1.7
SOO	3.7
SOP	0.7
DSTAG	2.4
MSTAG	32.9
TUTAG	64.7
Main FA (%)[a]	
Palmitic acid	11.8
Oleic acid	74.1
Linoleic acid	6.9
Linolenic acid	0.7
SFA	15.8
MUFA	77.6
PUFA	7.6
Quality parameters	
Free Acidity (%)	0.4
POV (meq O_2/kg of oil)	18.3

Data are expressed as mean of three determinations. RSD≤3.5%. [a]The results were expressed as normalized area in percent

MW samples exhibited a trend of values comparable to those previously reported for EVOO samples heated under similar conditions [19,31], with a slight decrease after 15 min of microwave heating, but this trend was quite different from that of OV heated oil. Previous experiments, carried out under similar conditions, have shown that EVOO temperature reached 120 °C after 90 min of OV heating with a further increase to 140 °C for longer time of treatment (by unpublished data of the authors). On the other hand, temperatures of EVOO were markedly higher at the end of MW heating; from about 180 °C (at 3 min) to reach 313 °C at the longest time of heating (15 min).[31] Thus, it could be hypothesized that OV and MW samples probably underwent quite different kinetics of thermo-oxidation, leading to a higher lipid degradation degree for the latter heated oils in comparison with the former samples. TAG products from higher stages of lipid oxidation (e.g. polymers) were previously found in olive oil subjected to comparable times of MW heating.[32]

Table 2 PAV number (n) of fresh and thermo-oxidized EVOO sample

Time (min)	PAV (n)
Fresh	5
OV	
30 min	8
90 min	20
120 min	27
180 min	34
360 min	61
900 min	102
1440 min	Nd
MW	
3 min	11
4.5 min	22
6 min	28
9 min	40
12 min	41
15 min	35

Data are expressed as mean of three determinations. RSD≤3.5%. *Nd* not determinable

DSC thermograms, obtained for untreated and thermally stressed EVOO sample, are reported in Figure 1 for MW samples (inserts a and b, for cooling and heating, respectively) and in Figure 2 for OV oils (inset A and B, for cooling and heating, respectively).

Generally, cooling curves are more interpretable than those obtained upon heating, as crystallization of oils is well known to be influenced by chemical composition, whereas the well-known melting-re-crystallisation phenomenon, named polymorphism, could easily occur during the melting of the original oil crystals.[5] EVOO showed the classical cooling profile with two exothermic events, the major (peak a of Figures 1a and 2a) peaking at lower temperature (T_p~−38 °C), which accounted for the crystallization of the majority of the most unsaturated lipid fractions and the minor (peak b of Figures 1a and 2a, T_p~−16 °C) at higher temperature, previously related to more saturated TAG, whose profile was also found to be influenced by minor components (diacylglycerols in particular).[6]

DSC profile of EVOO upon heating was more complex than upon cooling as this oil exhibited multiple transitions; a minor exothermic peak (peak c of Figures 1b and 2b, T_p at ~−15 °C) and, successively, two endothermic events (peaks d and e of Figures 1b and 2b, at ~−5 and 5 °C, respectively), whose profiles appeared to show some differences in relation to fatty acid composition, especially for the peak at the highest temperature (e).[23] The first event (c) at the lowest temperature regions of the thermogram may be attributed to an exothermic molecular rearrangement of crystals into more stable polymorphic forms, already observed in other vegetable oils,[5] whereas the two endothermic peaks at higher temperature (d and e), characterized by multiple overlapping contributions, could be ascribed to the melting of crystallized lipids.[33]

Thermal treatment induced changes in DSC profiles both upon cooling and heating. Upon cooling, the most evident change was a shift towards lower temperature and a

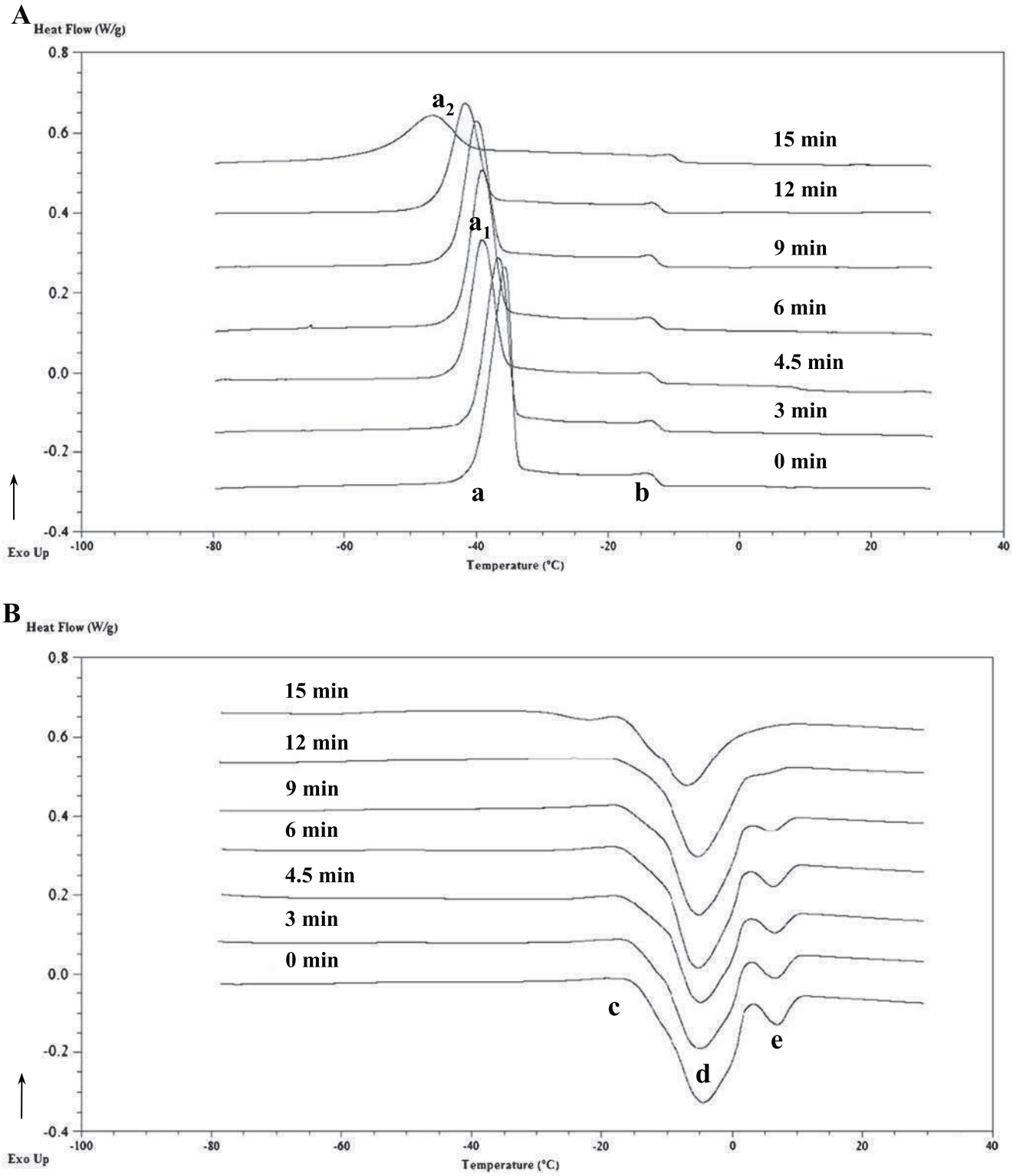

Fig. 1 Evolution of DSC cooling (**a**) and heating (**b**) thermograms for EVOO at different MW heating times. Main transitions are indicated with lowercase letters. Cooling thermograms: (*a*) major exothermic peak at time 0; (a_1) shifted peak a from 4.5 to 12 min of MW treatment; (a_2) shifted peak a after 15 min of MW treatment; (*b*) minor exothermic peak at time 0. Heating thermograms: (*c*) first exothermic transition at time 0; (*d*) major endothermic peak at time 0; (*e*) minor endothermic peak at time 0

decrease of height of the major exothermic peak (a) both for OV and MW samples up to 360 and 12 min of treatment (peak a_1 of Figures 1a and 2a), respectively, and in relation to PAV increase (Table 2). The profile of this peak was

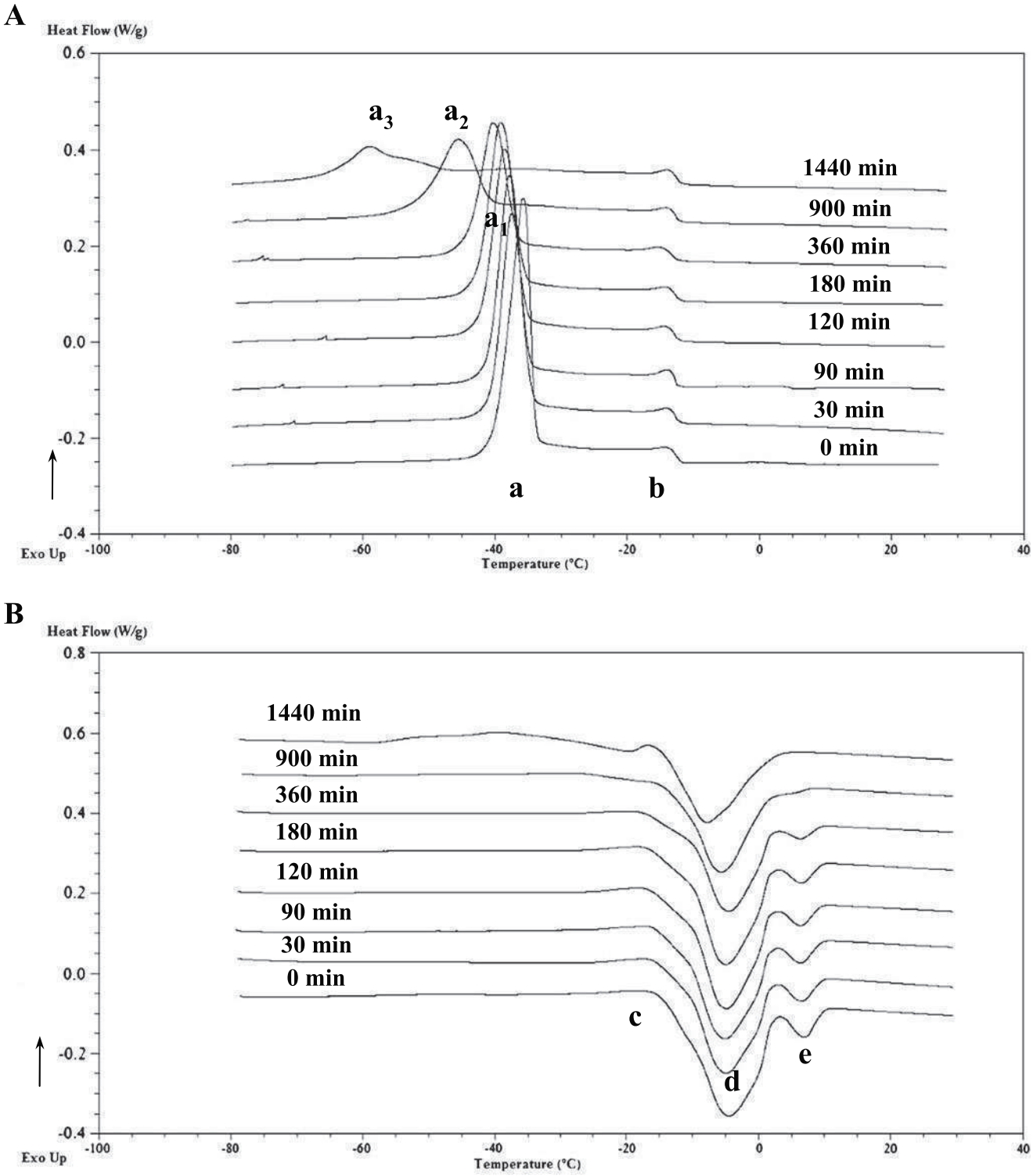

Fig. 2 Evolution of DSC cooling (**a**) and heating (**b**) thermograms for EVOO at different OV heating times. Main transitions are indicated with lowercase letters. Cooling thermograms: (*a*) major exothermic peak at time 0; (a_1) shifted peak a from 30 to 360 min of OV treatment; (a_2) shifted peak a after 900 min of thermal treatment; (a_3) shifted peak a after 900 min of thermal treatment; (*b*) minor exothermic peak at time 0. Heating thermograms: (*c*) first exothermic transition at time 0; (*d*) major endothermic peak at time 0; (*e*) minor endothermic peak at time 0

dramatically altered by both heating treatments, as this peak broadened with a consistent height decrease and a further shift towards lower temperatures up to −60 and −50 °C for OV (peak a_3 of Figure 2a) and MW (peak a_2 of Figure 1a),

respectively, significantly enlarging the range of transition. All these changes were previously observed upon DSC cooling for thermally-oxidized EVOO samples.[17–19] They could be attributable to the formation of lipid polar compounds, which were found not to crystallize in the DSC cooling range of analysis.[14] These molecules were reported to induce a shift of crystallization transition towards lower temperatures as well as to hinder the alignment of TAG molecules and to weaken their ability to come in contact by means of intermolecular bonding, causing a depletion of crystallizing lipid.[17–19,34]

The minor peak (b) was less altered by thermo-oxidation. Its profile remained unchanged for OV heated samples whereas it became less evident after 15 min of MW treatment, with the maximum skewed towards higher temperatures and a shift of onset temperature of crystallization from −11 to −8 °C in microwaved sample, at least. This shift may be attributable to other thermal degradation phenomena rather than oxidation, as the formation of hydrolysis molecules from TAG (e.g. diacylglycerols), which were previously found to influence onset temperature of crystallization [35] were reported for MW heated EVOO.[36]

Heating profiles were also altered and became more complex than the originals, as a consequence of thermo-oxidation (Figures 1b and 2b). To the authors' best knowledge, heating thermograms of conventionally thermo-oxidized EVOO were not reported in literature, yet.

Both thermal treatments caused a shift in the major endothermic peak (d) towards lower temperature after 90 min for OV and 4.5 min for MW, respectively, when PAV became about 4 times higher than the initial value (Table 2). In addition, peak height (d) decreased and dramatically changed its profiles after 900 and 15 min of treatment, respectively, with the appearance of shoulder peaks embedded. The minor endothermic peak (e) became less evident after 360 (OV) and 9 (MW) min of heating and disappeared at 900 (OV) and 15 (MW) min. Other endothermic/exothermic transitions appeared at the lowest temperature region of thermograms (from −60 °C to −20 °C), at the longest times of both treatments and apart from the original transition one (c).

The changes in heating profiles could be attributable to the formation of different and less stable polymorphic crystals than pure oil, probably related to the formation of mixed crystals with lipid oxidation products during crystallization.[37] These crystals became to melt at lower temperature being more easily disrupted by heat.[37] Lipid oxidation molecules may have also made the transition/rearrangement of TAG polymorphic crystals more difficult, dramatically changing phase transition profile (peak c) at the lowest temperature region of the thermogram for samples at the longest time of treatments, as previously observed for EVOO samples heated under MW.[18,19]

Application of PCA Models

PCA is one of the most used tools for data compression[38] and in this study, it was used in order to find the main DSC regions affected by thermo-oxidation that could be useful to tentatively discriminate samples according to different treatments and/or heating times. The analysis was performed on the whole digitised calorimetric curves avoiding complex procedure of extrapolation of thermal properties by thermograms.

In the first step, PCA was performed using the entire set of samples obtained in the experiment (0 min, OV samples and MW samples). PCA revealed that three principal components (PC1 to PC3) explained almost 87.21% of the total variance between the data. The scatter plots defined by the PC1 vs. PC2 and PC2 vs. PC3 components were reported in Figures 3a and b, respectively. It was observed that PC1 and PC2 were correlated to time exposure to heat (Figure 3a) discriminating among three groups of samples (named G1, G2 and G3), which were distinguished in relation to different times of treatment. G1 approximately accounted for short (from 30 to 180 min for OV and from 3 to 6 min for MW), G2 for medium (360 min for OV and from 9 to 12 min for MW) and G3 for long (G3, from 900 to 1440 for OV and 15 min for MW) heating times. These groups were also in relation to different thermo-oxidation degree, G1 exhibited PAV values lower than 35, G2 in the range 40–60, whereas G3 contained OV samples with PAV values higher than 100 and the MW heated oils that may have reached higher lipid oxidation stages than second group, but not measured by this oxidative stability index. In addition, control sample (0 min) appeared to be well resolved from the three groups above mentioned. On the other hand, OV and MW samples appeared to be not well separated by means of this PCA analysis, as shown in Figure 3b.

In order to be able to differentiate between these two heating treatments, the raw sample (0 min) was eliminated from the model, therefore reducing the variables involved and proceeding with a new PCA where only OV and MW oils were considered. In this case, the first three PC functions accumulated 91.15% of the variance explained, showing a better fitting of DSC data than the previous model. The scatter plots obtained with this PCA were reported in Figure 3c (PC1 vs. PC2) and 3D (PC2 vs. PC3). Figure 3c, showed that the 1st and 2nd PC scores were able to distinguish among the different heating time, too, preserving the separation into three groups according to heating time and oxidation stage. In addition, Figure 3d showed that the 3rd PC was able to separate among the different heating source. All OV samples presented score values higher than −1.5, whereas almost all MW samples had score values lower than cut value for 3rd PC, with the only sample at 6 min of MW treatment out of the model. This observation

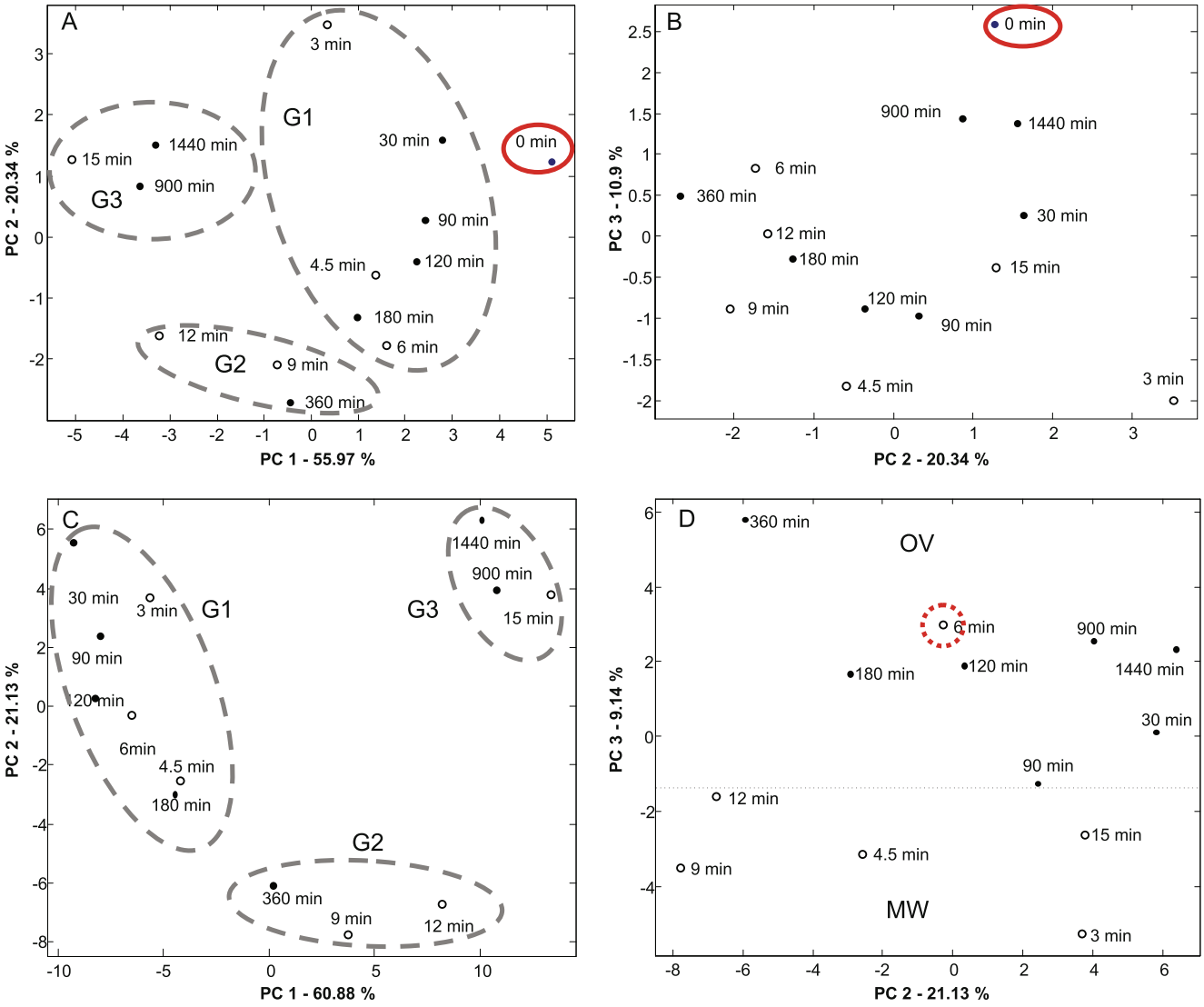

Fig. 3 First PCA plot showing MW(○) and OV (•) samples and heating time separation (*grey dashed lines*), control sample (0 min) is underlined with a *solid line red circle*; **a** 1st and 2nd scores; **b** 2nd and 3rd scores. Second PCA plot showing MW(○) and OV (•) samples and heating time separation (*grey dashed lines*), sample out of model is underlined with a *dashed line red circle*: **c** 1st and 2nd scores; **d** 2nd and 3rd scores

suggests that the 3rd component models could be strictly related to the type of heating process.

The loadings of these PC1, PC2 and PC3 were analysed in order to find the main regions of DSC thermograms involved in the group and/or treatment separations. A plot showing unfolded mean centred (MC) DSC signals of EVOO was shown in Figure 4a while the corresponding loading plots for PC1, PC2 and PC3 were shown in Figure 4b, c and d, respectively. The influence of the original variables (DSC heat flow) on PC score values could be better understood by means of these PC loadings plots. Positive loading values found in a defined DSC regions implied that a rise in this region has positive influence on score values. In the same way, a negative high loading value in a DSC region implied that a more positive value than the mean in this region have a negative impact on score values for this PC.

Figure 4b shows the loading of PC1. First loading appeared to be influenced by the signal diminution (negative loading values) of the major peak of lipid crystallization (transition a of Figures 1a and 2a, peaking at about −30 °C) and the increase in the signal at about −40 °C (transition a_1 of Figures 1a and 2a, positive values), which led to positive scores on PC1. This was related with the decrease of height and the shift towards lower temperature of the major exothermic peak (peak a_1 of Figures 1a and 2a), which accounted for the crystallization of the more unsaturated lipid fractions in the classical DSC cooling profile for EVOO.[6] Thus, G2 and G3 groups presented positive scores on PC1. On the other hand, 1st loading showed positive

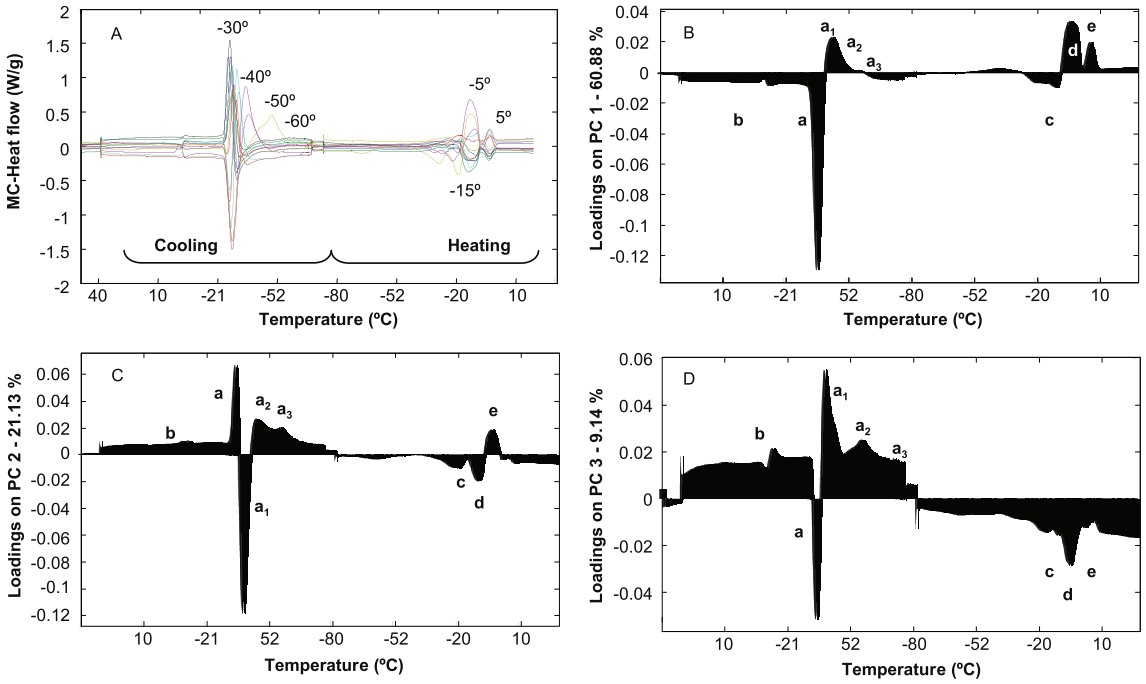

Fig. 4 Plot showing mean centred (MC) DSC signal (**a**) of EVOO and corresponding loading plots for 1st (**b**), 2nd (**c**) and 3rd PC (**d**). Cooling and heating regions of the thermograms were also indicated in insert a

values for peaks located at −5 and 5 °C, the two endothermic events (transitions d and e of Figures 1b and 2b) related to the melting of crystallized lipids [33] for the heating thermogram, which accounted for a decrease of signals of these two peaks also leading to positive scores on PC1 (Figure 4b), as a consequence.

Discrimination according to PC2 appeared to be more complex as samples belonging to G1 presented both positive (OV oils at 30 and 90 min of heating and MW sample at 3 min) and negative (OV at 180 min and MW at 4.5 and 6 min) scores, thus making a further separation within this group possible. Otherwise, among more thermo-oxidized samples, G2 presented high negative and G3 high positive scores, respectively. The shift in transition a (Figures 1a and 2a) to a_1 (Figures 1a and 2a) accounted for a high negative loading on PC2 (Figure 4c) leading to negative scores on this component not only for G2 samples but also for those oils of G1 that exhibited PAV values higher than 20 (Table 2). Positive loadings were found for peaks a_2 and a_3 (Figures 1a and 2a). These peaks were associated with a more marked shift in the major lipid crystallization transition (peak a of Figures 1a and 2a) and related to an higher level of lipid oxidation as for samples of G3 group, which presented high positive scores on PC2 (Figure 4c). Positive loading for peak a (Figures 1a and 2a) and negative loading for peak d (Figures 1b and 2b) led to positive scores on PC2 for samples from G1 group (oils with the lowest PAV values). Meanwhile, this component exhibited correlations with an increase in the intensity of the exothermic peak c (Figures 1b and 2b) at −15 °C of the heating thermograms (negative loading and positive scores on PC2) that were exhibited by samples of G3 groups, and a decrease in the intensity of peak at −5 °C (transition e of Figures 1b and 2b) for the heating curve (positive loading on PC2 and positive scores).

On the 3rd PC component (PC3), which is correlated with the discrimination according to the heating treatments (Figure 3d), there was a great contribution of baseline curve both for heating and cooling profiles. The highest discrimination between the two heating treatments was placed in the overall enhancement of the DCS baseline signal for OV samples that also presented the highest score values on this PC (Figure 3d). This enhancement was related to the changes in whole cooling and heating transition profiles (Figures 2a and 2b) that were markedly altered especially at the highest treatment times. Likewise, high positive loading values for peaks a_1, a_2 and a_3 (shift in peak a of Figures 1a and 2a) were also evident, being this loading contributions more evident for OV heating where the shift in the peak a was more pronounced.

Conclusions

In conclusion, these preliminary findings give rise to the adoption of a novel strategy for the treatment of DSC data as digitised calorimetric curves could be chemometrically analysed and explored for the evaluation of thermal stress on EVOO.

Thermo-oxidized oil samples were differentiated by PCA according to changes in main DSC signals both by cooling and heating profiles. In particular, the shift in the major exothermic transition in cooling thermograms towards lower temperature and the decrease of height of the major endothermic transition of heating thermograms appeared to be most helpful to discriminate samples according to three different groups of treatment time (short, medium and long) by means of PC1 and PC2. These three groups also accounted for a different degree of thermo-oxidation in relation to PAV values. A discrimination was also possible taking into account different heating processes by means of PC2 and PC3.

In conclusion, the adopted procedure may be usefully employed to select appropriate heating conditions to be applied in relation to both the degree of thermo-oxidation and the EVOO composition. Further studies will be carried out in order to evaluate discriminant capability of the proposed strategy not only on EVOO but also on other vegetable oils with different chemical composition on the basis of the oxidative status reached by oils under the common thermal conditions used in industrial processing and food catering sectors considering several chemical stability indices.

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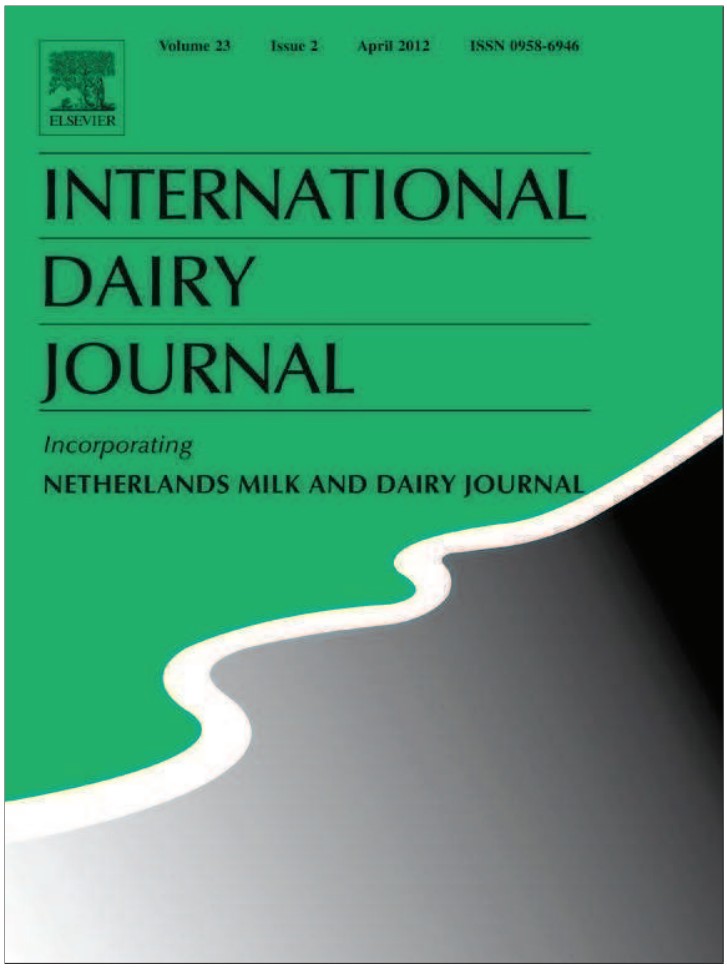

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Discrimination of grated cheeses by Fourier transform infrared spectroscopy coupled with chemometric techniques

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ABSTRACT

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), combined with chemometric analysis, was used to classify grated Parmigiano-Reggiano cheese from other grana-type cheeses (so called for their granular texture) from Italy, central and northern Europe. A total of 36 grated cheese samples (21 Parmigiano-Reggiano and 15 Italian and non-Italian non-Protected Designation of Origin) were analysed. Samples were scanned in the range of 4000–700 cm^{-1}. Two attenuated total reflectance accessories were utilised. Linear discriminant analysis (LDA) and principal component analysis were used to analyse spectral data after applying a moving windows algorithm for wavelength selection. Both methods successfully classified the four classes of grated cheese samples, and LDA was found to be the best chemometric approach. ATR-FTIR spectroscopy coupled with LDA is a promising technique, and merits further investigation as a reliable and rapid classification tool that does not require chemical analyses for discrimination of cheese.

1. Introduction

In recent years, many traditional and artisanal food products have been increasingly rediscovered by consumers as they are considered beneficial for daily nutrition due to their genuineness and authenticity. In the hard cheese, or "grana-type" category (so called for their granular texture), there are a large number of cheeses that have remained unchanged over time, maintaining the link with tradition and local methods of production. This linkage is the result of the interaction between natural and human factors, namely animal fodder and the technology used for production (Karoui & De Baerdemaeker, 2007).

One such case is the Protected Designation of Origin (PDO) Parmigiano-Reggiano cheese, one of the oldest traditional cheeses produced in Italy (Zannoni, 2010). The factors that make this product unique include the local fodder and pasture consumed by the dairy cows where any type of silage is forbidden, the quality of milk (Summer et al., 2003), the use of raw and partly skimmed cows' milk, supplementation with a natural whey starter before curd formation (Bottari, Santarelli, Neviani, & Gatti, 2010; Gala et al., 2008), the exclusive use of calf rennet and the prohibition of preservatives such as lysozyme, the use of copper vats, cheese master know-how (Bellesia et al., 2003), and aging for 12–36 months.

Parmigiano-Reggiano, as well as a wide number of grana-type cheeses, can be sold as packaged pieces or grated; the former are easily recognizable because the emblem embossed on the rind certifies its origin (Regattieri, Gamberi, & Manzini, 2007). However, when grated, there may be some doubt about composition and authenticity. Therefore, it is necessary to have the analytical means of verifying that the origin of a grated cheese corresponds with its labelling.

The development of novel and sophisticated analytical techniques have been used for determination of authenticity and geographical origin of various foodstuffs, including dairy products (Luykx & van Ruth, 2008; Reid, O'Donnell, & Downey, 2006). In particular, Fourier transform infrared spectroscopy (FTIR) combined with multivariate data analysis such as principal component analysis (PCA), Fisher discriminate analysis (FDA), linear discriminate analysis (LDA), support vector machine, k-nearest neighbour and partial least squares have all been successfully used to monitor the quality parameters or authenticity of olive oil (Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010; Maggio et al., 2009), honey (Hennessy, Downey, & O'Donnell, 2008), fruit

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(Vardin, Tay, Ozen, & Mauer, 2008), wine (Tarantilis, Troianou, Pappas, Kotseridis, & Polissiou, 2008), meat (Argyri, Panagou, Tarantilis, Polysiouand, & Nychas, 2010) and dairy products (Nicolaou, Xu, & Goodacre, 2010). With a special concern towards cheeses, FTIR coupled with chemometrics has been used to investigate the composition of aged cheese (Subramanian, Alvarez, Harper, & Rodriguez-Saona, 2011), sensory and texture parameters (Blazquez et al., 2006), geographic origin (Karoui et al., 2004; 2005) and aging time (Lerma-García, Gori, Cerretani, Simó-Alfonso, & Caboni, 2010; Martin-del-Campo, Picque, Cosio-Ramirez, & Corrieu, 2007).

Moreover, FTIR is a non-invasive and non-destructive technique that offers a fast and cost effective method of food analysis (Karoui et al., 2004), and can be easily applied in research, quality control laboratories and industrial settings. One of the main advantages of the FTIR spectrometer is the ability to scan spectra (4000–700 cm^{-1}) directly on the cheese sample by collecting an interferogram of a sample signal with an interferometer, which measures all infrared frequencies simultaneously. The spectrum recorded thus provides a "fingerprint" of each cheese sample since it contains information about its physicochemical characteristics (Coates, 2000).

In this study, attenuated total reflectance (ATR) FTIR spectroscopy combined with PCA and LDA was used to discriminate grated Parmigiano-Reggiano cheese from other grana-type cheeses from Italy, central and northern Europe, with the purpose of establishing a fast, highly reproducible and non-invasive method for quality control of grated cheese. Since the non-homogeneity of components in various cheeses is well-known (McQueen, Wilson, Kinnunen, & Jensen, 1995), a further aim of this investigation was to test the reproducibility of the analytical method with two type of ATR accessories: an ATR accessory used for liquids that permits analysis of 1.5–2 g of sample, and an ATR accessory used for the analysis of 0.5–1 g of solid sample.

2. Material and methods

2.1. Grated cheeses

A total of 36 plastic-sealed grated cows' milk cheese samples were used for FTIR analysis. The cheese samples were manufactured in Italy, the Czech Republic and Lithuania, and classified as follows: 21 Parmigiano-Reggiano at 12 and 24 months of age, and 5 Italian non-PDO, 5 Czech non-PDO and 5 Lithuanian non-PDO that were aged for unknown time. Parmigiano-Reggiano cheese samples were kindly donated by the Cheese Consortium (CFPR), whereas others were purchased in blocks from local markets. Each sample were grated with an industrial toothed grater roller prior to the direct determination of the content of fat, protein, moisture and salt with a Foss FoodScan analyzer (FoodScan Lab, Type 78800, FOSS, Hillerød, Denmark) by the CFPR personnel. The grated samples were then kept in a sealed box until analysis to prevent changes in relative composition due to moisture loss. The origin and status of conservation of all samples were guaranteed by the suppliers.

2.2. FoodScan calibration

The instrument calibration was carried out by introducing into the ISIscan management software of FoodScan (Routine Analysis Software, FOSS), a data set consisting of spectral data and analytical data obtained by wet chemistry analysis, such as moisture (drying the sample at 105 °C for 6–8 h), fat, protein and salt (Careri, Spagnoli, Panari, Zannoni, & Barbieri, 1996). A brief description of the data set is shown in Table 1. After calibration, a periodic external validation was carried out which evaluates parameters obtained by the wet chemistry analysis and FoodScan for 20 samples (data not shown). In all cases no significant statistical differences between both methods were observed.

Table 1
FoodScan parameters for calibration and validation procedures of moisture, fat, protein and salt content.

Parameter	Moisture	Fat	Protein	Salt
Calibration				
Number of samples	197	165	237	812
Mean	34.1	29.2	31.8	1.31
SD[a]	4.2	2.4	2	0.44
Calibration range	21.3–46.8	21.9–36.5	25.53–38.0	0.00–2.64
Internal validation				
SEC[b]	0.3323	0.3686	0.4640	0.0665
R^2	0.9939	0.977	0.9501	0.9776
SECV[c]	0.3531	0.3958	0.4613	0.0697

[a] Standard deviation.
[b] Standard error of calibration.
[c] Standard error during cross validation.

2.3. Instrumentation and spectral acquisition

Spectra were acquired using a Tensor 27 FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid interferometer and a DigiTect detector system, and coupled to an ATR accessory. Two ATR accessories were tested for the purpose of verifying the intensity of the absorption and the reproducibility of the measurements: an ATR for analysis of solid food matrices (MIRacle accessory, Pike Technologies, Madison, WI, USA) coupled to a MIRacle high-pressure clamp, and an ATR accessory for the analysis of liquid matrices (Specac Inc., Woodstock, GA, USA) with a larger surface of contact and path length. The latter was adapted for the analysis of solid samples by using an ad-hoc clamp and a Teflon form to allow good contact of the sample against the crystal. Both ATR accessories were equipped with a ZnSe reflection crystal. Analyses were carried out at room temperature, and spectra were acquired (32 scans per sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 software (Bruker Optics).

2.4. Sample treatment and data acquisition

Prior to analysis, each sealed box containing grated cheese was shaken vigorously. Samples were treated differently depending on the ATR accessory used. For ATR accessories destined for liquid sample analysis, about 1.5–2 g of grated cheese was spread with a spatula across the cell. The cheese was worked gently with the spatula to ensure complete coverage. The cell was then closed with a Teflon form while exerting pressure by the ad-hoc clamp to allow complete contact of the grated cheese against the crystal. Three spectra were recorded for each portion.

For the ATR accessories destined for solid sample analysis, three aliquots of grated cheese (10 g) were taken from the bottom, the centre and the surface of the box, and 0.5–1 g from each aliquot was deposited on the ATR surface and a pressure clamp was applied to obtain a thin cheese layer. Each aliquot was measured in triplicate. In both the cases, the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in n-hexane and then rinsed with acetone.

2.5. Chemometric techniques

LDA is a probabilistic classification technique that searches for directions (canonical variables) with maximum separation among

categories (Massart et al., 1998); the first canonical variable is the direction of maximum ratio between inter-class and intra-class variances. Classification of spectral data was carried out after applying a moving windows algorithm strategy for wavelength selection (Xiaobo, Jiewen, Povey, Holmes, & Hanpin, 2010) as a feature selection technique. This evaluates the performance of LDA classification on the basis of predictive ability in the cross-validation groups (internal prediction rate), and searches for the range of variables with the largest classification success. Performance of LDA classification was evaluated on internal prediction rate in the cross-validation procedure for each class.

A PCA is concerned with explaining the variance–covariance structure of a set of variables through linear combinations of these variables. The general objectives are data reduction and thus simplification of interpretation. PCA was carried out after applying the moving windows algorithm strategy for wavelength selection (Jolliffe, 2002) as a feature selection technique. Performance of PCA classification was evaluated on the basis of predictive ability in the cross-validation groups (internal prediction rate). All chemometric techniques were carried out using MATLAB 7.0 (The Mathworks, Natick, MA, USA, 2007).

2.6. Data processing

LDA and PCA were carried out on the spectral data. ATR-FTIR spectra were standardized by using mean centre pre-treatment to remove or minimise any unwanted baseline contribution. Data matrices were reduced by taking a minimal number of spectral point that allowed effective discrimination; only one of ten spectral points was used for the calculations (reducing the calculation time). To apply classification methods, grated cheese samples were divided into four classes: class 1 (Parmigiano-Reggiano), class 2 (Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

3. Results and discussion

3.1. Grated cheeses composition

The content of fat, protein, moisture and salt was determined by near infrared transmission in the range of 1050–850 cm^{-1}, and the composition is reported in Fig. 1. The Parmigiano-Reggiano grated cheeses were produced by the approved method of the CFPR that is restricted to grated cheese having the technical and technological parameters as reported in the regulation (Council Regulation, 1992). Nevertheless, the information about the content of fat, protein, moisture and salt was insufficient to verify the affiliation of each grated cheese sample to their group, and to discriminate the Parmigiano-Reggiano from the others grated cheese groups.

3.2. Choice of the ATR accessory

To assess analytical performance, two ATR accessories were tested. This phase was necessary to evaluate which of the two better met the requirements of repeatability, and was therefore able to best represent the sample. The Pike MIRacle accessory coupled to the clamp permits analysis of a small and constant amount of solid food matrix on which a uniform force is exerted, so that the sample adheres to the crystal as a thin film. On the other hand, the Specac accessory was conceived for analysis of liquid food matrices, and therefore has no clamp to distribute the sample as the liquid spreads itself on the crystal, although it is possible to measure higher quantities of sample due to the larger surface of the crystal.

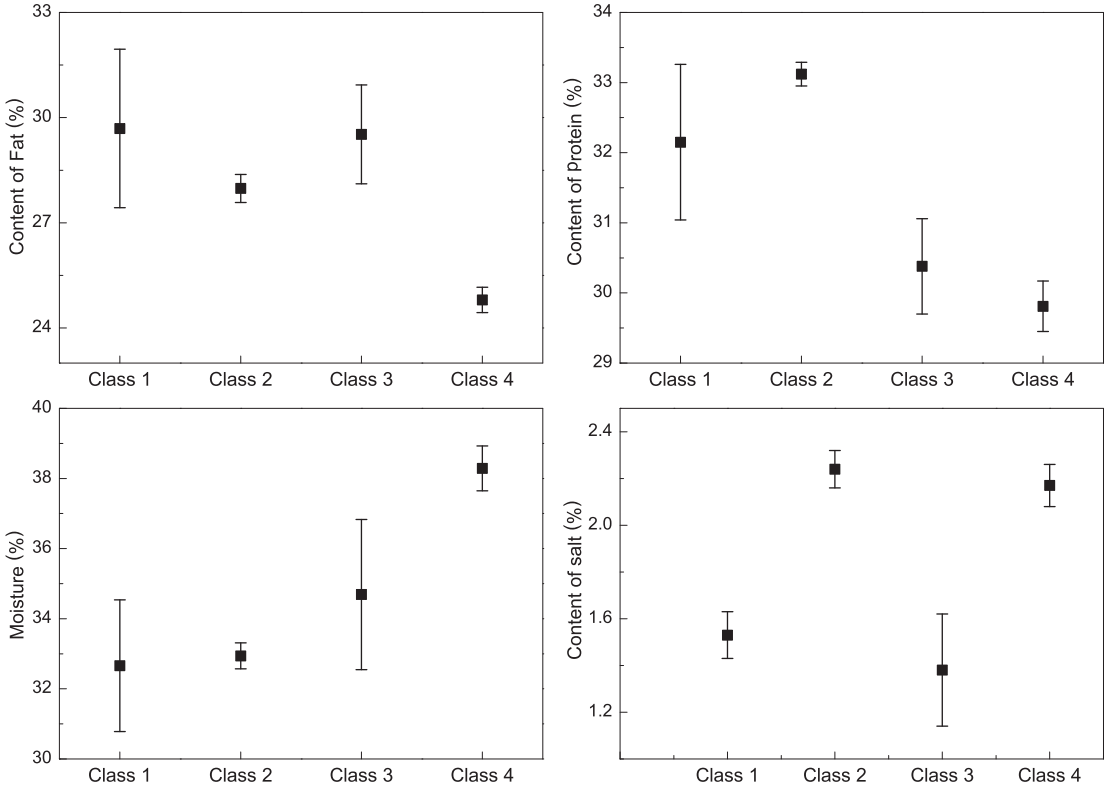

Fig. 1. Mean values (square symbols) and standard deviation (bars) of fat, protein, moisture and salt content of the grated cheeses: class 1 (Parmigiano-Reggiano), class 2 (Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

Fig. 2 shows a typical ATR-FTIR spectrum and the estimated background noise of a grated cheese sample, acquired with the two ATR accessories. For this procedure a random sample of Parmigiano-Reggiano was measured ten times using each ATR. The mean spectrum and the standard deviation of absorbance were calculated at each wavelength.

The mean spectra acquired with the ATR accessory for solid food matrices generally showed a lower absorbance than the ATR accessory for liquid food matrices, which was higher due to the larger surface of the crystal, however, the estimated background noise was always lower for the ATR accessory for solid food matrices, probably due to homogeneous pressure applied by the clamp on a small surface. Therefore, considering the decreased inter-sample variation as the most robust classification model, for analysis of grated cheese samples, the Pike MIRacle accessory coupled to the high-pressure clamp was adopted.

3.3. FTIR data and chemometric analysis

FTIR spectra were dominated by several typical peaks. Absorbance in different wavenumber ranges could be assigned to the following contributions: –O–H stretching in hydroxyl groups (3700–3000 cm^{-1}), –C–H stretching in fatty acids (3000–2800 cm^{-1}), –C=O of acids and esters (1750–1650 cm^{-1}), amide I and amide II of proteins (1650–1450 cm^{-1}), esters and aliphatic chains of fatty acids (1460–1150 cm^{-1}) and C=O and C–C stretching of acids (1200–800 cm^{-1}) (Karoui et al., 2005; Lerma-García et al., 2010). Additionally, the bands between 1450 and 1410 cm^{-1} include absorbance from acidic amino acids, such as glutamic acid, and the aliphatic chains of fatty acids (Subramanian et al., 2011).

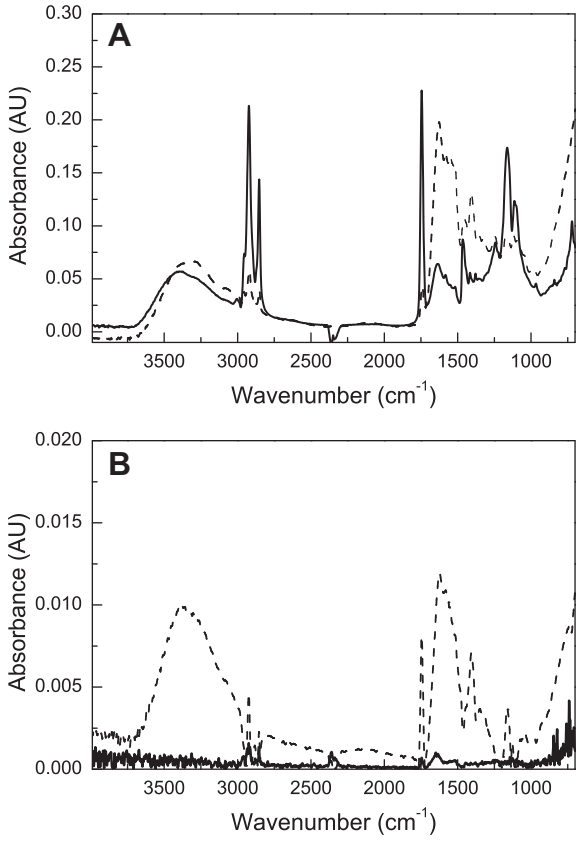

Fig. 2. Mean IR spectra overlay ($N = 10$) of Parmigiano-Reggiano grated cheese (A) and instrumental background noise (B) using an ATR accessory for solid (—) and liquid samples (- - -).

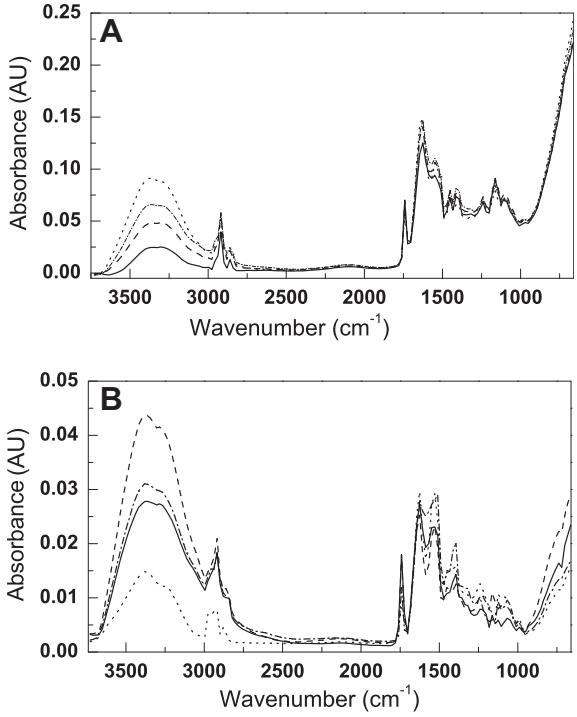

Fig. 3. Mean FTIR spectra using the ATR accessory for solids (A), and intra-class absorbance variation (B) of four grated cheese samples: Parmigiano-Reggiano, $N = 21$ (—); Italian non-PDO, $N = 5$ (- - -); Czech non-PDO, $N = 5$ (···) and Lithuanian non-PDO, $N = 5$ (—·—).

Monitoring multiple functional groups simultaneously offers advantages over other spectral methods, thus favouring its use for rapid analysis by spectroscopy. However, as shown in Fig. 3A, the spectral data of the four classes of cheese, Parmigiano-Reggiano, Italian non-PDO, Czech non-PDO and Lithuanian non-PDO, did not reveal obvious differences from visual inspection according to the origin of cheese. Additionally, the regions with higher differences among classes also had higher variations within the same class, as can be seen in Fig. 3B.

A chemometric method, namely the PCA model, was applied to detect differences between samples (Table 2), although classification procedures were not acceptable for analytical purposes. The best results were obtained in the full spectral range for class 1 (Parmigiano-Reggiano) with a correct classification of 68%. Class 2 (Italian non-PDO) was the worst class, achieving a prediction rate of 48%, thus demonstrating that PCA was not able to group these grated cheeses according to origin.

Table 2 and Fig. 4 show the results obtained by LDA classification in the calibration and cross-validation procedures. The moving windows algorithm strategy for wavelength selection was applied in order to obtain the best spectral range for the classification. This

Table 2
Results of classification for principal component analysis (PCA) and linear discriminant analysis (LDA) and predictive ability in cross-validation procedures.

Class	Classification ability (%)		Prediction ability (%)	
	LDA	PCA	LDA	PCA
Class 1	100	68	99	68
Class 2	100	48	100	48
Class 3	100	59	100	61
Class 4	100	60	100	60
Means	100.0	59.1	99.9	59.6

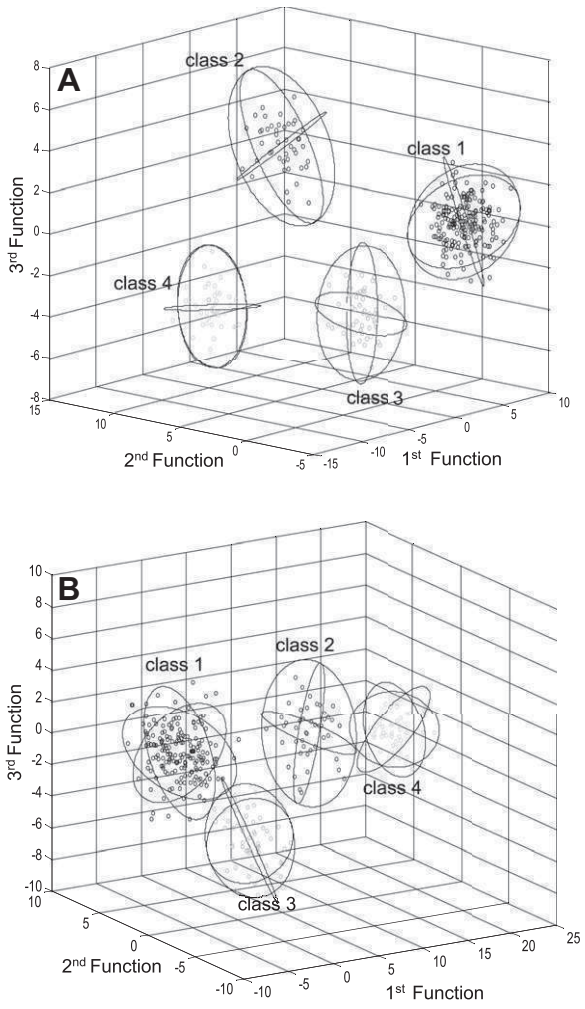

Fig. 4. Three-dimensional score plots of discriminant analysis for (A) calibration and (B) cross-validation determined by discriminant factors 1, 2 and 3 for mid-infrared spectra (3361.7–1317.3 cm^{-1}) for grated cheeses with four different origins: class 1 (Parmigiano-Reggiano), class 2 (Italian non-PDO), class 3 (Czech non-PDO) and class 4 (Lithuanian non-PDO).

strategy evaluated the performance of LDA classification by a cross-validation procedure along all spectral range, varying systematically the initial wavelength and width of the window. The best results were obtained in the 3361.7–1317.3 cm^{-1} spectral range. The percentages of correct classification and prediction were 100% and 99%, respectively. In this case, the worst in terms of classification was class 1 (Parmigiano-Reggiano) with a correct prediction rate of 99%, also demonstrating that the LDA results are in agreement with the origin of grated cheese samples.

4. Conclusions

In this investigation, a total of 36 grated cheese samples were discriminated according to origin by ATR-FTIR. Two ATR accessories were used to evaluate reproducibility of FTIR spectra obtained from non-homogenous samples. The ATR accessory for solid food matrices was chosen to assess minor variations in inter-sample classes. Spectral data was analysed using two different chemometric approaches: PCA and LDA. The LDA method was successfully applied to spectral data after used the moving windows algorithm strategy for wavelength selection. LDA-FTIR analysis had a predictive classification ability approaching 100% during the cross-validation procedure whereas PCA could not successfully classify the different types of cheese. The results showed that LDA-FTIR is a promising technique that could potentially be a reliable and fast classification tool for PDO grated cheeses. Lastly, such a rapid analysis of grated cheese may provide information on overall differences between hard cheeses, which could complement results from other analytical identification and quantification methods.

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A New Patented System to Filter Cloudy Extra Virgin Olive Oil

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Abstract: The aim of this investigation was to evaluate the chemical and sensory quality of three extra virgin olive oils (EV1-3) subjected to a new patented system to clarify cloudy oils through the insertion of an inert flow gas (argon) in olive oil mass. For this purpose, several quality parameters were determined on the three clarified (EVC1-3) and untreated (EVNC1-3) samples. In particular, the system patented by the University of Bologna and Sapio (a private company that supplies gas for industrial and research sectors) was applied to a 50 L batch of each oil after its production by a low-scale mill. The EV samples were bottled and stored at room temperature and kept in darkness before analysis. Basic quality indices including free acidity, peroxide value, specific absorption in the conjugated triene region and sensory analysis, as well as the composition of the major (fatty acids) and minor (tocopherols, polar phenols, volatiles, water) compounds were determined after three months of storage. The oxidative stability under stress conditions was also assessed. The main results concern the higher overall quality of the EV samples clarified by the patented system compared to untreated ones. The quantity of water significantly decreased in all clarified samples. Above all, the non-clarified oils showed a tendency to quickly develop off-flavors over time and to decrease their oxidative stability.

Keywords: Clarified edible oil, extra virgin olive oil, filtration systems, inert gas, minor compounds, quality parameters.

INTRODUCTION

Newly produced extra virgin olive oil (EV), obtained solely by mechanical and physical processes, is a turbid and opalescent juice having suspended solid impurities as traces of water in micro-emulsion and dispersed protein type material and mucilages, which can promote enzymatic activity and compromise chemical and organoleptic quality.

The filtration process step of a cloudy EV is generally carried out before storage in order to remove suspended solids and humidity and to make the oil more clear and brilliant for the bottling phase. The most widely used traditional filtration systems in the olive oil industry are filter aids based on inorganic (diatomite) or organic fibrous materials such as cellulose or cotton introduced among the filter plates of the filter press equipment. In recent years, a cross-flow filtration system has been also proposed as an interesting and alternative method that involves oil flow parallel to the membrane instead of in a perpendicular flow in conventional method, using membranes with different characteristics in terms of pore sizes [1-3]. During filtering, quantitative and qualitative changes take place, especially on minor components [4]. In particular, a decrease in polar phenols, molecules of great importance for oil stability and human health [5], has been demonstrated [4, 6-9]. Some traditional filtration systems can also involve problems derived from the disposal of filtering materials. In order to solve these problems and at the same time guarantee the quality level of EV, innovative filtration systems have been recently proposed. In this regard, Italian researchers have developed two new filtration processes: the first, developed by Filterflo (Binasco, Milan, Italy) and previously applied to the enology sector [10], consists in the use of a filter bag system (usually polypropylene) in which the EV is directly transferred from storage tanks to filtration equipment and across the filter bag, allowing removal of suspended solids and a satisfactory level of oil limpidity [11]. The second system, patented by the University of Bologna and Sapio [12] is based on the flow of an inert gas (nitrogen or argon) that is introduced directly in the center of the olive-oil mass. Gas insertion generates a circular movement of the oil mass that facilitates separation of the suspended solids with a significant clarification effect and water decrease. In a recent publication [11], very similar and satisfactory results in terms of chemical composition and sensory characteristics in EV treated by polypropylene filter bag and argon flow gas were reported. Additionally, it is important to underline that the clarification by an inert flow gas avoids the use of organic materials from coming into contact with the olive oil. In order to confirm these first and positive results, in the present work the effect of the patented system, in particular using argon as an inert flow gas, on the chemical and sensory quality of three bottled EV samples compared to non-clarified ones was studied.

MATERIALS AND METHODS

Samples

The analyzed samples were monocultivar EV, all obtained from Correggiolo olives. The olives were harvested in

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the Emilia-Romagna region (Italy) in autumn 2011 at three different ripening levels (25 October, 9 November and 28 November) and processed separately by continuous low-scale plants (Oliomio 150, Tavernelle Val di Pesa, Florence, Italy) equipped with a hammer crusher, vertical malaxator and a two-phase decanter, in order to produce three different samples (EV1-3). 100 kg of olives were processed for each batch and aliquots of the obtained oils were clarified using a filtration system, based on argon gas flow (EVC1-3), while the other aliquots were not clarified (EVNC1-3). The filtration equipment, based on the flow of an inert gas, consisted of a filter tank and inert gas tank developed and patented by University of Bologna and Sapio [12]. Cloudy virgin olive oil was placed in the 50 L filter tank at room temperature and an insertion device for inert gas was connected to the bottom to the tank. The flow rate of argon gas, injected directly into the center of the olive oil mass, was 15 L min^{-1}. All EV samples were stored in several dark glass bottles (0.5 L) at room temperature and kept in darkness before analysis. Analytical determinations started at least 3 months after the production of the samples (from the beginning of February) and the analytical plan was completed within 3 months. All analytical determinations were performed on oil withdrawn from the core of the bottle.

Chemicals

All chemicals were of analytical reagent grade and purchased from Fluka, Sigma-Aldrich (Steinheim, Germany), such as the standards alpha-tocopherol, gallic acid and 4-methyl-2-pentanone.

Basic Quality Parameters

Free acidity, peroxide value and ultraviolet spectrophotometric index (K_{270}) were evaluated according to the official methods described in the EEC Reg. 2568/91 of the Commission of the European Union [13]. All analyses were determined in triplicate for each sample.

Sensory Analysis

Sensory analysis was performed according to the EC Reg. 640/2008 [14] by a fully trained group of 8 expert tasters. A set of positive (bitter, pungent, fruity and other pleasant attributes such as leaf, grass, artichoke, tomato, almond, apple, others) and negative (winey-vinegary, fusty-muddy, mouldy, rancid, others) sensory attributes were evaluated using a continuous scale from 0 to 10 cm related to the perception of flavor stimuli, according to the judgment of assessors. The median and the robust standard deviation (EC Reg. 640/08) were calculated for each attribute. If the value of the robust standard deviation was higher than 20%, the sensory analysis was repeated. For statistical analyses the mean values were considered (n=3).

Fatty Acid Composition

The fatty acid composition of oil samples was determined as the corresponding methyl esters (FAMEs) by gas chromatography (GC) (Clarus 500 GC Perkin Elmer Inc., Shelton, CT, USA) analysis. FAMEs were prepared by alkaline treatment carried out by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol, according to Christie [15]. Chromatographic conditions were previously described by Bendini *et al*. [16]. Results were expressed as % of FAME on the total amount of FAMEs (n = 3). The FAMEs were grouped in three different categories, according to the specific number of double bonds: SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, PolyUnsaturated Fatty Acids. Moreover, the OA/LA (ratio between oleic acid and linoleic acid) was calculated for each sample.

OSI (Oxidative Stability Index)

The oxidative stability under forced conditions was determined using an eight-channel oxidative stability instrument (OSI) (Omnion, Decatur, IL). The OSI time was determined according to the analytical protocol described by Jebe *et al*. [17]. Briefly, samples (5 g) were loaded onto each channel and heated at 110 °C under atmospheric pressure. At this stage, the air flow was injected into the center of the sample mass to bubble through the oil at 150 mL min^{-1}, generating an increase in conductivity due to formation of short-chain volatile acids. This increase, measured in distilled water channels directly connected to the sample, determined an induction period (OSI time), expressed in hours and hundreds of hours (n=3).

Determination of Tocopherols

For the quali-quantitative determination of tocopherols, 0.3 g of sample was dissolved in 10 mL of isopropanol and passed through a 0.45 μm filter before HPLC analysis. The chromatographic separation of these compounds was performed on a 150 mm × 4.6 mm i.d., 120 Å, Cosmosil π NAP column (CPS Analitica, Milan, Italy). The injection volume for HPLC was 20 μL. The mobile phases used were methanol:water (90:10 v:v) having water acidified with 0.2% phosphoric acid in isocratic gradient as eluent A and acetonitrile as eluent B to wash the column. The flow rate was 1 mL min^{-1}, and analyses were made at room temperature. The total run time was 35 min. Separated tocopherols were quantified with a DAD detector at 292 nm. A calibration curve was calculated by using six points of alpha-tocopherol at different concentrations, estimated from the amounts of the analytes in samples, and was linear over the range studied (r^2 = 0.999). Results were given in mg of alpha-tocopherol per kg of oil (n=3).

Liquid-liquid Extraction of Phenolic Compounds

Extraction of phenolic compounds from EVOOs was performed following the protocol proposed by Pirisi *et al*. [18] and modified according Rotondi *et al*. [19]. Three replicates were carried out for each sample. The extracts for the spectrophotometric assays were stored at -18 °C before use.

Spectrophotometric Assays: Total Phenols (TP) and o-diphenols (o-DPH)

The TP and *o*-DPH of the extracts were determined using a UV-Vis 1800 Shimadzu spectrophotometer (Kyoto, Japan) and evaluated according to Singleton *et al*. [20] and Mateos

et al. [21], respectively. TP and *o*-DPH were detected at 750 and 370 nm, respectively, and quantified using gallic acid calibration curves (r^2 = 0.993 and r^2 = 0.998, respectively). Data were expressed as mg of gallic acid per kg of oil.

Chemical Index of Bitterness (K_{225})

Chemical evaluation of bitterness of polar phenolic extracts (n=3) was carried out spectrophotometrically at 225 nm using a UV-Vis 1800 Shimadzu spectrophometer (Kyoto, Japan) [22, 23].

Determination of Water Amount (WA)

The water amount was analyzed with a TitroMatic 1S instrument (Crison Instruments, S.A.; Alella, Barcelona, Spain) according to the method described by Gomez-Caravaca *et al*. [4]. Clarified and unclarified EV were dissolved in a solution of chloroform/Hydranal-solvent 2:1 (v/v), and the titrating reagent (Hydranal-Titran 2) was added until the equivalence point was reached. Each sample was introduced three times, and the quantity of the sample was measured with the back-weighting technique. The quantity of water was expressed as mg of water kg^{-1} of oil (n = 3).

Determination of Volatile Compounds

Volatile compounds were identified and quantified by SPME-gas chromatography coupled with quadrupolar mass-selective spectrometry, according to Baccouri *et al*. [24]. The results were expressed as mg of internal standard (4-methyl-2-pentanone) per kg of oil (n=3).

Statistical Analysis

The software XLSTAT 7.5.2 version (Addinsoft, USA) was used to elaborate chemical and sensory data by Analysis of Variance (ANOVA) and Principal Components Analysis (PCA) Before PCA analysis, the data were standardized, normalized and centred.

RESULTS

As detailed in the Materials and methods section, all samples were obtained from a low-scale mill working in continuous system but without a final cleaning of virgin olive oil by a vertical centrifugation as applied in several industrial plants. Therefore, aliquots of newly produced cloudy oils were subjected to clarification by the patented system based on argon flow in a suitable steel tank and then bottled, whereas other aliquots were bottled without any treatment. Consequently, for all samples, the lipid matrix during the storage remained in contact with the dispersed particulate that tended to settle to the bottom over time (see Fig. **1**). It must be emphasized that the experimentation entailed use of non-optimal conditions of storage for the overall quality of an extra virgin olive oil. However, this allowed investigation of the possible effects of clarification by argon flow on the analytical parameters checked. In Fig. (**1**), it can be observed that the patented system led to good separation of the initially dispersed particulate as a thick sediment on the bottom.

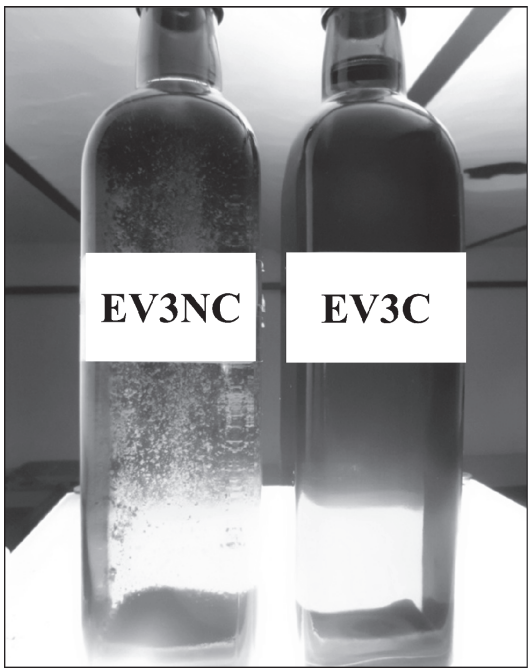

Fig. (1). Bottles of clarified (EV3C) and non-clarified (EV3NC) extra virgin olive oils, obtained from Correggiolo olives harvested at the third ripening level.

As shown in Table **1**, according to basic chemical parameters, all samples belonged to the extra virgin olive oil category, as the mean values were within the legal limits for free acidity (FA), peroxide value (PV) and specific extinction at 270 nm (K_{270}). The clarified oils (EV1-3C) did not show any critical differences compared to the nonclarified ones (EV1-3NC) both in terms of hydrolysis and oxidative degradation as suggested by the results of free acidity percentages and peroxide values (primary oxidation products) as well as absorptions in triene conjugated spectrum region (secondary oxidation products).

As already reported in a previous work [11], the clarification process by argon flow had no substantial effect on percentages of the main fatty acids as oleic, palmitic, linoleic, stearic and linolenic (single data not shown) that cover the usual range in olive oils. In fact, the compositions in fatty acids were characterized by high contents in monounsaturated (MUFA) and low in polyunsaturated (PUFA) fatty acids as also seen by the oleic (OA) and linoleic (LA) acids ratios. Small differences among the three pairs of clarified and non-clarified samples were also found as a slight reduction of this ratio was obtained due to the increase of degree of ripening of Correggiolo olives (from EV1 to EV3).

The most important lipophilic phenols quantified by HPLC-DAD in EV samples were alpha and gamma tocopherols. Experimental data showed slightly higher values of both tocopherols in clarified samples (data not shown). As a consequence, the results concerning the TOT TOC also revealed a tendency towards loss of these molecules in the non-clarified oils.

The clarification system did not affect the total polar phenolic content, the sum of all hydrophilic molecules having a phenol group (TP) or all compounds with catecholic

Table 1. Mean values of analytical parameters in oil samples after three months of storage. EV1-3C, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening were clarified by the patented system with argon gas and bottled; EV1-3NC, monovarietal extra virgin olive oils obtained from Correggiolo olives were harvested at three different degrees of ripening and bottled without previous clarification. FA, Free Acidity expressed as g of oleic acid 100 g^{-1} of oil (≤ 0.8 % is the legal limit for extra virgin olive oil); PV, Peroxide Value expressed as meq of oxygen kg^{-1} of oil (≤ 20 meq O_2 kg^{-1} is the legal limit for extra virgin olive oil); K_{270}, specific extinction at 270 nm (≤ 0.22 is the legal limit for extra virgin olive oil); SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, PolyUnsaturated Fatty Acids; OA/LA, ratio between Oleic Acid and Linoleic Acid; TOT TOC, Total amount of Tocopherols expressed as mg of α-tocopherol kg^{-1} of oil; TP, Total Polyphenols by spectrophotometric assay expressed as mg of gallic acid kg^{-1} of oil; *o*-DPH, total amounts of ortho-diphenols by spectrophotometric assay expressed as mg of gallic acid kg^{-1} of oil; K_{225} evaluation of bitterness as specific absorption at 225 nm by spectrophotometric assay; WA, water amount valued by Karl-Fisher automatic titration expressed as mg kg^{-1}; ALD C6, aldehydes with six carbon atoms produced by the lipoxygenase pathway expressed as mg of 4-methyl-2-pentanone kg^{-1} of oil; TOT VOL, total amount of volatile compounds expressed as mg of 4-methyl-2-pentanone kg^{-1} of oil; OSI, time of oxidative stability calculated by Oxidative Stability Instrument and expressed in hours and hundredth of hour. Different letters in the same row indicate significant differences (Fisher LSD, $p < 0.05$).

	EV1C	EV1NC	EV2C	EV2NC	EV3C	EV3NC
Basic quality parameters						
FA	0.3 b	0.3 b	0.4 a	0.5 a	0.5 a	0.5 a
PV	10 e	8 f	14 b	15 a	11 d	12 c
K_{270}	0.11 b, c	0.10 c	0.11 b	0.11 b	0.15 a	0.16 a
Fatty acid categories						
SFA	17.9 a	17.3 a,b	17.0 b, c	17.1 b	16.3 c	17.3 a, b
MUFA	74.1 a	74.1 a	72.6 b	72.9 b	72.5 b	71.7 c
PUFA	7.8 e	8.1 d	9.7 c	9.7 c	10.3 a	10.2 b
OA/LA	9.2 b	9.3 a	7.3 c	7.3 c	6.9 d	6.9 d
Minor components and oxidative stability						
TOT TOC	317 a	273 b	187 d	185 d	271 b	263 c
TP	218 a	236 a	232 a	221 a	204 a	207 a
o-DPH	495.9 a, b	512.9 a	498.1 a, b	484.7 b	474 b, c	455.6 c
K_{225}	0.30 a	0.26 b	0.28 a, b	0.28 a, b	0.31 a	0.26 b
WA	622 c	875 b	238 e	990 a	433 d	901a, b
ALD C_6	7.5 b, c	0.9 d	7.8 a, b	0.1 d	11.3 a	3.9 c, d
TOT VOL	20.5 a	10.7 b	14.6 b	13.3 b	20.0 a	20.4 a
OSI	25.1 a	23.4 b	18.8 c	14.4 d	14.0 e	13.5 f

structure (*o*-DPH). Bitterness is an important sensory attribute of virgin olive oil, usually assessed by tasting and related to phenolic compounds. As suggested by some authors [22, 23], this parameter can be also estimated by the spectrophotometric measurement of the specific absorbance at 225 nm (K_{225}) carried out on the phenolic fraction extracted by the EV sample.

There was a good agreement between the bitterness evaluated by both sensory and chemical tests as shown by the data in Tables **1** and **2** and in Figs. (**2 a-c**).

As expected, and in agreement with previous results [11], for all three pairs of samples the highest water content (WA, Table **1**) was recorded in non-clarified virgin olive oil. This effect seems to be linked to oxidative stability (OSI), and in fact, for all three couples of oils, better stability in terms of oxidative degradation was shown by the clarified oil compared to the untreated one.

Considering the aldehydes with six carbon atoms (ALD C_6) formed by the lipoxygenase enzyme pathway (LOX), which are known to be particularly important for the perception of pleasure green notes in extra virgin olive oils [24], it must be underlined that there was a significantly higher presence of this volatile fraction in oil samples subjected to clarification with argon gas, bottled and stored for few months.

From the data shown in Table **2** and sensory profiles in Figs. (**2 a-c**), there was a clear tendency for the non-clarified

Table 2. Mean values relative to the sensory attributes perceived in samples and evaluated on a 10 cm scales (using the sheet of the Reg. EU 640 2008). EV1-3C, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening and clarified by the patented system with argon gas before bottling; EV1-3NC, monovarietal extra virgin olive oils obtained from Correggiolo olives harvested at three different degrees of ripening and bottled without clarification. Different letters in the same row indicate significant differences (Fisher LSD, $p < 0.05$)

	EV1C	EV1NC	EV2C	EV2NC	EV3C	EV3NC
Positive attributes						
Fruity	3.5 a	1.9 c	3.1 a, b	2.4 b, c	2.7 a, b	2.3 b, c
Almond	2.9 a	1.4 b-d	2.4 a, b	1.2 b-d	2.3 a-c	0.6 d
Bitter	4.4 a	1.6 c	3.2 b	3.0 b	3.8 a, b	3.2 b
Pungent	4.4 a	1.5 c	4.1 a, b	3.1 b	4.3 a	3.6 a, b
Negative attributes						
Fusty-Muddy	0.2 b	1.4 a, b	0.6 a, b	1.2 a, b	1.5 a, b	1.8 a
Winey-Vinegary	0.0 b	1.2 a	0.0 b	0.5 b	0.6 a, b	0.0 b

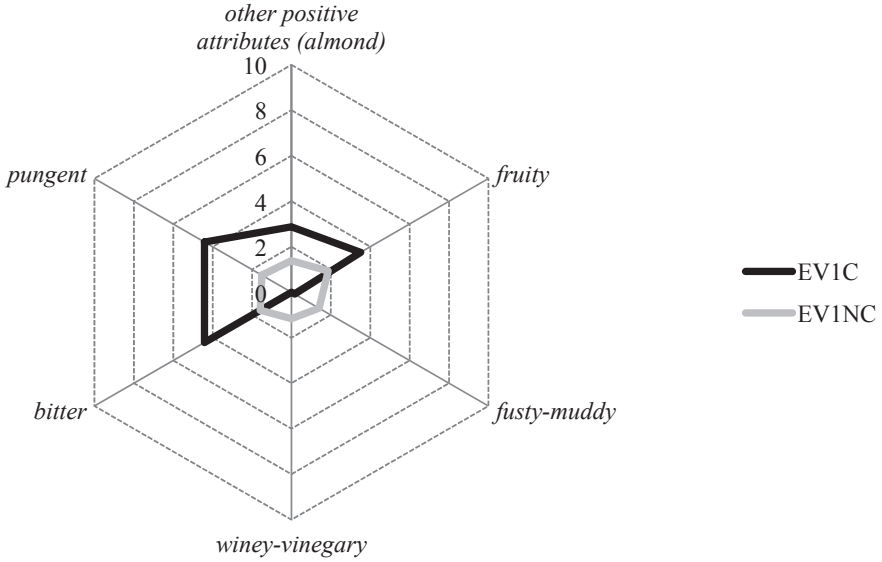

Fig. (2 a). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV1C (clarified) and EV1NC (non-clarified) pair (see materials and methods section for a description of samples).

oil samples to quickly develop off-flavors over time, in particular fusty-muddy and winey, due to degradation of proteins and sugars present in micro-dispersion into the oil.

In contrast, the corresponding clarified samples were less disqualified for these sensory defects and richer in fruity and almond intensities, probably as a consequence of the sudden deposit of micro-dispersed residuals.

Fig. (**3**) shows the biplot graph obtained from the principal component analysis built using several chemical (Table **1**) and sensory oil characteristics (Table **2**). In general, a PCA picture shows comparison of multidimensionally expressed sensory/chemical product attributes, projected on a two-dimensional surface, described by orthogonal factors used as dimensions: principal components PC1 and PC2. Percentages indicate what % of evaluated product variability is related to each PC. The first two components explained 83.98% of the total variance (55.44% for PC1 and 28.54% for PC2). The sensory attributes and chemical parameters are shown as vectors, the mutual direction of attribute/parameter vectors indicates positive correlation if they are close to each other and go to the same direction, while negative correlation is seen if they are close but in an opposite direction, or unrelated when they are perpendicular. Concerning the location of products on the PC1/PC2 surface, if they are close to each other it means that those products are similar (taking in account a combination of all evaluated attributes), while if they are far away from each other they differ strongly. Approximate position of the product near certain attribute/chemical parameter vector(s) allows for the conclusion that the product has this attribute/chemical parameter particularly expressed. For the construction of Fig. (**3**), two positive (almond and pungent) and two negative (winey-vinegary and fusty-muddy) sensory attributes as well as two chemical pa-

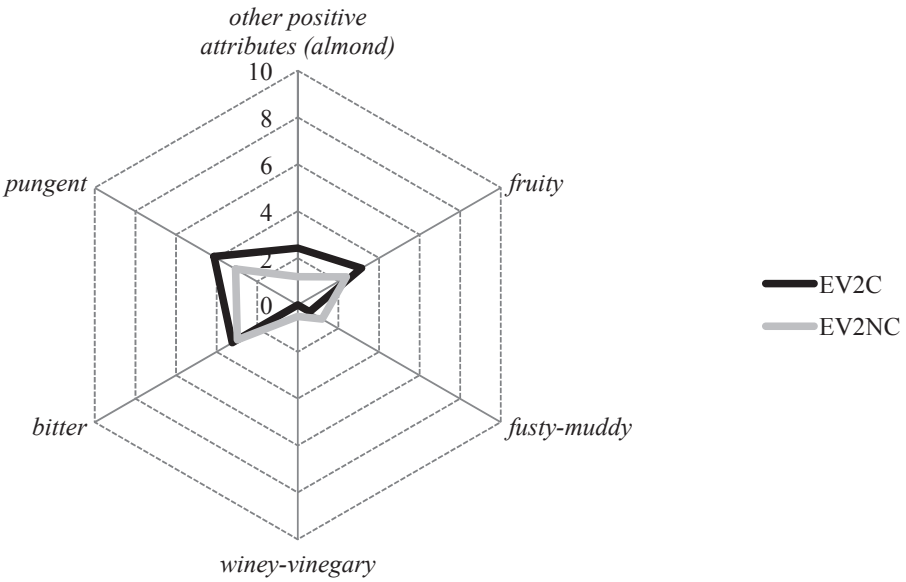

Fig. (2 b). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV2C (clarified) and EV2NC (non-clarified) pair (see materials and methods section for a description of samples).

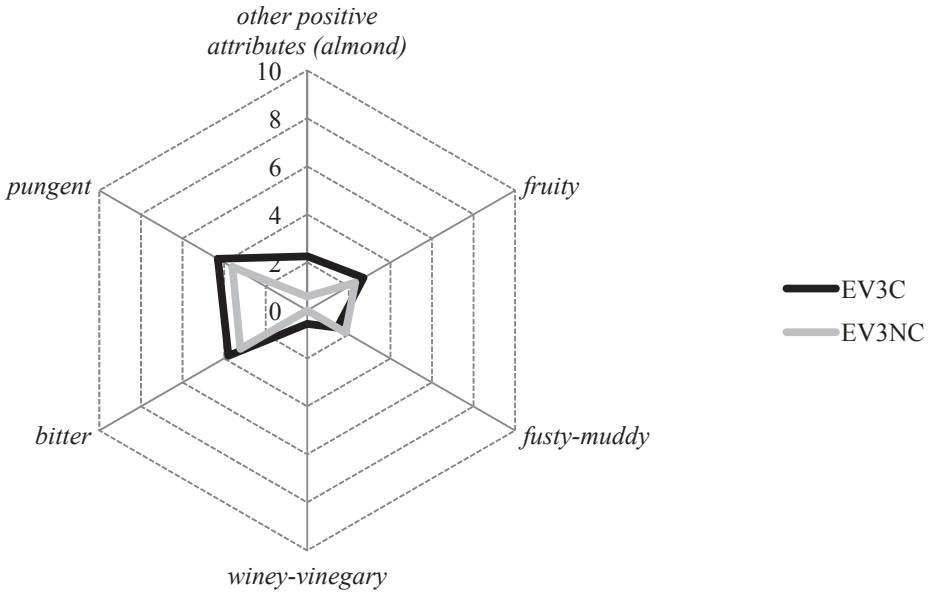

Fig. (2 c). Spider web graph of the sensory attributes (expressed as mean values calculated on the 10 cm scales) of the EV3C (clarified) and EV3NC (non-clarified) pair (see materials and methods section for a description of samples).

rameters (OSI and WA) were chosen. Only parameters with enough high F values (test LSD Fischer, $p<0.05$) and which were not redundant were chosen. In particular, the two pairs EV2C/EV3C and EV2NC/EV3NC located, respectively, in the 2nd and 3rd quadrants were characterized, above all, by the low or high intensities of off-flavors. In fact, the EV2NC/EV3NC pair was disqualified for negative attribute known as fusty-muddy perceived by assessors as defective for the presence of the unpleasant note known as fusty-muddy, whereas the EV2C/EV3C pair did not show the same negative descriptor. Concerning the EV1C/EV1NC couple, EV1C (in the first quadrant) was the best sample in terms of sensory characteristics with a high intensity of almond note (a secondary positive note characteristic of Correggiolo extra virgin olive oils), whereas the EV1NC (in the fourth quadrant) was disqualified by off-flavors recognizable as fusty-muddy and mostly winey-vinegary.

Considering Pearson's correlations, the almond descriptor was positively related to the other pleasant ones as fruity ($r = 0.829$, $p<0.05$). Similarly, the two positive sensations pungent and bitter were highly related to each other ($r = 0.945$, $p<0.05$). Total phenols and *ortho*-diphenols, well known to be mainly responsible for the bitter and pungent sensations in EV [5], also showed a high correlation ($r = 0.879$, $p<0.05$).

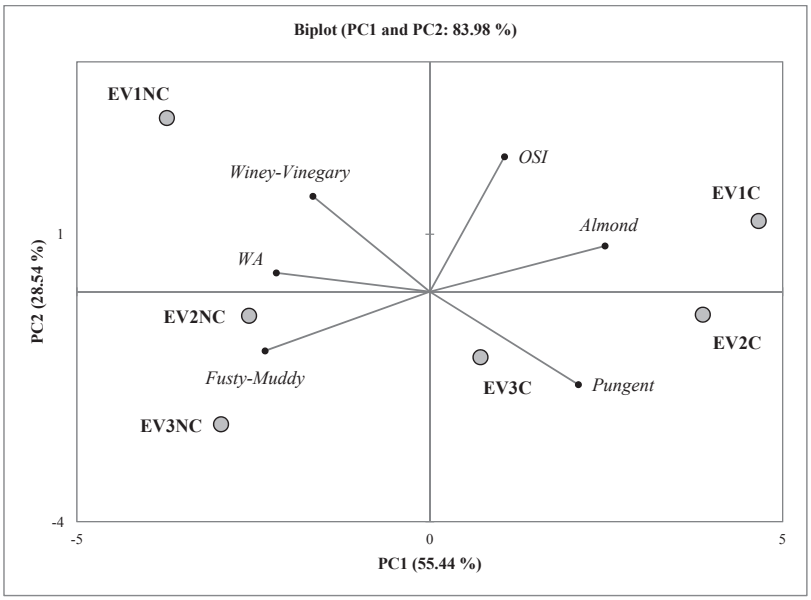

Fig. (3). Biplot graph obtained from the principal component analysis built using the selected chemical and sensory variables.

DISCUSSION

In general, non significant differences were found in clarified oils compared to the untreated ones in terms of hydrolysis and oxidative degradations, and all samples were within the legal limits for the extra virgin olive oil category. The clarification process with argon flow had no substantial effect on the fatty acid composition or total amount of polar phenolic compounds. Probably, during the first months of oil storage, these latter were protected by the presence of tocopherols that, in fact, showed a slightly lower concentration in non-clarified samples. A trend of a greater resistance to forced lipid oxidation for clarified samples compared to untreated oils was observed. This could indicate a greater stability of oils subjected to the argon flow technique prior to storage, perhaps as a consequence of a partial removal of oxygen in the oil mass. It is known that EV oxidative stability is influenced by different physical (temperature, exposure to light, presence or absence of oxygen) and chemical (composition in fatty acids and minor compounds) parameters. The highest values of the OA/LA ratio, in particular observed for oil samples produced by olives harvested at an early degree of ripening (EV1) together with the high content of minor antioxidant compounds, above all tocopherols (TOT TOC), can explain their higher time of oxidative stability (OSI). A high positive Pearson's correlation ($r = 0.947$, $p<0.05$) was found between the OA/LA ratio and OSI values. In the non-clarified oils, a tendency towards loss of TOT TOC was evident. This could be explained assuming that consumption of these antioxidant compounds was able to inhibit the development of the first step of the lipid oxidation [16]. In this regard, a significant negative correlation ($r = -0.833$, $p< 0.05$) was seen between TOT TOC and PV. In agreement with previous results [11], the quantity of water significantly decreased in all clarified samples, and thus the patented system using argon gas can be considered effective in reducing the water content.

Nonetheless, there was a clear tendency for non-clarified oils to quickly develop off-flavors over time, in particular fusty-muddy and winey, and this may be due to degradation of proteins and sugars present in the micro-dispersion. On the other hand, the application of the patented system permitted a rapid deposition of this material on the bottom of the bottles as shown for the EV3C/EV3NC couple (Fig. **1**). Indeed, there was a significant decrease in water content in all three clarified samples compared to the non-clarified oils, which resulted in protection of the positive sensory attributes fruity and almond notes, which are linked to the presence of specific aldehydes with six carbon atoms. In fact, a negative and significant Pearson correlation ($r = -0.844$, $p<0.05$) was found between ALD C_6 and WA.

It can be assumed that the clarified virgin olive oils were less disqualified for sensory defects than the untreated samples as also suggested by the biplot graph obtained from principal component analysis. At the same time, it is important to emphasize that the results of the volatile content and sensory analysis confirmed that clarification of cloudy EV using a flow of inert gas does not reduce the intensity of the main organoleptic attributes that contribute to consumer acceptance.

CONCLUSION

The proposed system permits clarification of extra virgin olive oil, avoiding any contact of the oil with organic or inorganic filtering material. The treatment appears to be very suitable and sustainable for the extra virgin olive oil category, which is declared by law as "…oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil…" [13]. As demonstrated in this short report and the previous investigation by Lozano-Sanchez *et al*. [11], treatment with nitrogen or argon allows persistence of positive attributes in unfiltered oils that are generally more perishable than filtered ones due to the presence of higher quantities of moisture and suspended materials. Moreover, this treatment maintains freshness longer, with less development of off-flavors. This effect could be related to three events: the first is the

quicker separation of moisture and the precipitate from the oil, due to gas bubbling. This phenomenon causes a reduction of the contact area and thus a partial separation from the oil (deposition) of the possible sites of fermentation. The second event is the lowering of the oxygen dissolved in the oil, which is partially washed out/substituted with nitrogen or argon, while the third, and probably the most important, is the depletion of the dispersed water. In fact, it is true that the clarified oil, as previously observed [11], was significantly less rich in water (by around one third and one half, respectively, when nitrogen and argon are used) compared to untreated oils. Furthermore, even if the visible material of the clarified and non-clarified oils has not yet been compared, which will be a focus of future studies, it is reasonable to suppose that the oil separated on the bottom by clarification is also significantly less rich in water, compared to that softly in suspension as in the non-clarified oil (easily visible in Fig. **1**). These three phenomena may help to explain the reason why the clarified oil is less inclined to develop defects over time.

CURRENT & FUTURE DEVELOPMENTS

For an industrial mill, in terms of cost the choice of nitrogen or argon cannot be ignored: nitrogen gas costs about 1 Euro per cubic meter, while argon gas is around three times more expensive. The prices of the same gases in the liquid state, which are less bulky for transportation, but purchasable only for companies with a high work capacity, are also lower even though argon is still about three times more costly (around 0.4 Euro and 1.2 Euro per cubic meter, respectively, for nitrogen and argon).

In the future, some improvements of the patented system need to be made: firstly, a better geometry of the bottom of the oil tank permitting easy discharge of the precipitate and its removal from the clarified oil will be studied, and a vertical window needs to be inserted for better control of turbidity. Secondly, taking into account the possible different sizes of tanks in which to apply the clarification system, correct proportions between the cloudy oil mass and the inert flow rate need to be defined. Finally, possible modifications to the system could help to define the optimal relation between uniformity of gas bubbling, changed by adjusting the rotation speed supply, as well as to reduce its consumption in order to obtain a more compact cake at the bottom of the tank.

CONFLICT OF INTEREST

The authors declare no competing financial interest or conflict of interest.

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LIST OF ABBREVIATIONS

EV	=	extra virgin olive oil
EVC	=	clarified extra virgin olive oil by the patented system
EVNC	=	not clarified extra virgin olive oil
FA	=	Free Acidity
PV	=	Peroxide Value
K_{270}	=	specific extinction at 270 nm
SFA	=	Saturated Fatty Acids
MUFA	=	Monounsaturated Fatty Acids
PUFA	=	Poly-Unsaturated Fatty Acids
OA/LA	=	Oleic Acid and Linoleic Acid ratio
TOT TOC	=	Total Tocopherols
TP	=	Total Polyphenols
o-DPH	=	Total ortho-diphenols
K_{225}	=	specific absorption at 225 nm
WA	=	water amount
ALD C_6	=	aldehydes with six carbon atoms
TOT VOL	=	Total Volatiles
OSI	=	Oxidative Stability Index

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Original article

Detection of low-quality extra virgin olive oils by fatty acid alkyl esters evaluation: a preliminary and fast mid-infrared spectroscopy discrimination by a chemometric approach

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Summary A set of eighty-one extra virgin olive oils (EVOOs) was analysed according to the new quality parameters relative to the total amount of methyl and ethyl esters of fatty acids [Σ (FAMEs + FAEEs)] and the ratio between ethyl and methyl esters [ratio of FAEEs/FAMEs (RFF)]. Acquisition of the mid-infrared spectra was also performed by Fourier Transform Infrared Spectroscopy (FT-IR). Chemical and spectroscopy data were chemometrically elaborated, and FT-IR coupled by Partial Least Square (PLS) methodology was developed. Results were statistically similar to official procedure in terms of analytical performance for Σ (FAMEs + FAEEs) and RFF in EVOOs: a good agreement between predicted and actual values on calibration data sets was found (0.98 and 0.83, respectively) and the limit of quantification was low enough (29.3 mg kg^{-1}) considering the actual limits for Σ (FAMEs + FAEEs). This new approach, time-saving and environmentally friendly, can be considered as a useful tool for screening procedures.

Keywords Extra virgin olive oil, fatty acid alkyl esters, FT-IR spectroscopy, low-quality oils., mildly deodorized olive oils.

Introduction

Extra virgin olive oil (EVOO) is characterised by one of the highest economic value in comparison with other vegetable oils, thanks to its well-known nutritional and sensory qualities (Velasco & Dobarganes, 2002). Unfortunately, EVOO is also easy to falsify: because of its prestige, it has always been illegally mixed with cheaper and low-quality oils (Harwood & Aparicio, 2000), especially to obtain EVOO sold in supermarkets and discount stores at low cost (Bendini *et al*., 2009a). The so-called lampante low-quality olive oils cannot be used as raw foodstuff for direct human consumption, as they have an acidity level that is too high, and their volatile profile is characterised by 'soft' off-flavours, derived from low-quality olives or from inappropriate procedures during oil extraction or storage. Deodorization applying a 'mild' technology, developed under vacuum and at low temperature, is able to remove unacceptable defects (mainly winey-vinegary, fusty-muddy sediment and musty), avoiding the formation of chemical traces in the oils exploited as forensic proof of fraud (Cerretani *et al*., 2008). In the last decades, several analytical methods have been proposed to detect such low-quality EVOOs and their admixtures, such as the determination of diacylglycerols and pheophytin (Serani & Piacenti, 2001a; Serani *et al*., 2001b), the amount of water present in the micro-emulsion of the oil and study of the volatile profile, especially taking into account of the ratio between ethanol and *E*-2-hexenal (Cerretani *et al*., 2008; Bendini *et al*., 2009a). In addition to the proposed analytical methods, one of the most reliable techniques seems to be determination of fatty acid methyl and ethyl esters (fatty acid alkyl esters, FAAEs) as methyl esters of fatty acid

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(FAMEs) and the ethyl esters of fatty acids (FAEEs) which are present in the waxy fraction of olive oils (Mariani & Fedeli, 1986; Mariani *et al*., 1991, 1992; Mariani & Bellan, 2008). In good quality EVOOs, FAMEs and FAEEs are present in very small amounts, while they are present in higher amounts in virgin, lampant olive oils (Mariani & Bellan, 2011) and in second-olive processing oil (the so-called repaso) (Cerretani *et al*., 2011). Actually, these compounds are formed as a consequence of degradation and fermentation processes of low-quality olives, which can be overripe, damaged or simply poorly preserved before they are processed (Biedermann *et al*., 2008). These alterations lead to a production of short chain alcohols by the degradation of the pectins by endogenous pectin-methyl-esterases (methanol) and the aerobic metabolism of microorganisms (ethanol) (Biedermann *et al*., 2008). At the same time, lipolysis of triacylglycerols with liberation of free fatty acids may occur. In these conditions, the formation of FAMEs and FAEEs by esterification can take place: this reaction is catalysed by the temperature reached during the 'mild' deodorization step (Perez-Camino *et al*., 2008), while it does not seem to occur during the storage of high-quality EVOOs (Mariani & Bellan, 2011). In reality, these molecules have two different but complementary meanings: first they are related to the olive (and consequently the olive oil) quality (Biedermann *et al*., 2008), and FAAEs can be considered as 'virtual' markers of possible 'mild-deodorization', as they resist and are not removed by this illegal treatment. In fact, the contemporary presence of a high level of FAAEs, without a clearly perceivable sensory defect, can be reasonably explained by the application of 'mild-deodorization'. Different analytical methods have been performed for the determination of FAMEs and FAEEs in VOO in recent years: the older ones are done by solid phase extraction (SPE) (Perez-Camino *et al*., 2008), while some modifications have been added (Bendini *et al*., 2009b and Cerretani *et al*., 2011). As of April 2011, the determination of alkyl esters, first proposed by the IOC (COI/T.20/DOC. NO. 28, 2009), became an official method adopted by the European Community law (EC Reg. 61/2011 and corrigendum). This method is based on solid–liquid chromatography (LC) by traditional glass column for isolating the fraction containing alkyl esters and waxes, with the aim to assign the evaluated sample to the commercial category of EVOO. Indeed, for EVOO, the concentration of the sum of FAMEs and FAEEs [Σ (FAMEs + FAEEs)] cannot exceed 75 mg kg^{-1} (EC Reg. 61/2011 and corrigendum). If Σ (FAMEs + FAEEs) is between 75 and 150 mg kg^{-1}, the oil can be considered as EVOO only if the ratio of FAEEs/FAMEs (RFF) is ≤ 1.5 (EC Reg. 61/2011 and corrigendum). It is interesting to underline that the European law permits a higher amount of alkyl esters for EVOO (between 75 and 150 mg kg^{-1}) only if RFF is lower or equal than 1.5, as the FAMEs are typically formed with the technological transformation of overripe olive fruits (Biedermann *et al*., 2008).

The determination of food authenticity and the detection of adulteration are current problems of increasing importance in the food industry. EVOO adulteration was extensively studied because it is a high added value product and adulteration employs more sophisticated methods nowadays (Arvanitoyannis & Vlachos, 2007). Traditionally, the chemical treatments of the samples required for determining authenticity of EVOOs are complex, expensive, time-consuming and tedious. On the contrary, FT-IR is a highly useful molecular spectroscopy technique because it is rapid, non-destructive, simple to perform and does not require sample pre-treatment. The employment of several multivariate methods [like principal component analysis, canonical analysis, linear discriminant analysis, cluster analysis, partial least squares (PLS), and surface response methodology] has become a prerequisite for several applications related primarily to food quality control in terms of authentication/adulteration, thanks to a substantial simplification of the classification/grouping task (Tzouros & Arvanitoyannis, 2001). Among the possible analytical approaches, the Fourier transform mid-infrared (FTIR) spectroscopy combined with multivariate chemometric procedures has been used by several authors for predicting the level of authentication/adulteration in EVOO samples based on chemical composition. This approach has been apply to correctly discriminate among genuine and adulterated olive oils containing soybean, corn, olive pomace oils or between pure EVOOs and the same oils adulterated with sunflower oil or with refined oils or with walnut or hazelnut oil (Arvanitoyannis & Vlachos, 2007; Özdemir & Öztürk, 2007; Gurdeniz & Ozen, 2009; Lerma-García *et al*., 2010; Maggio *et al*., 2010). Several chemometric approaches were used to treat variables of olive oil samples to classify extra virgin and ordinary olive oil samples and partial least-squares regression (PLS) resulted in higher prediction success rates (Tzouros & Arvanitoyannis, 2001). Nevertheless, to the best of our knowledge, there are no investigations regarding the application of this technique for a quick check of quality limits for EVOOs introduced by the new regulation. For this aim, the total amount of methyl and ethyl esters [Σ (FAMEs + FAEEs)], and the ratio between ethyl esters and methyl esters (RFF) of a set of eighty-one EVOO samples, sold in Italian supermarkets, were evaluated and compared with the limits proposed by the EC Reg. 61/2011 and corrigendum. Next, correlation between these chemical parameters and spectroscopy data of the oils was performed to detect low-quality EVOOs with a statistical model.

Materials and methods

Samples

A set of eighty-one EVOO samples was purchased from local supermarkets, where they were sold (and labelled) as EVOOs at low cost. The samples were bought in two different years, and the method used to evaluate the alkyl esters followed the historical evolution of the method, first reported in the literatures and then adopted by the European Community law (EC Reg. 61/2011 and corrigendum).

Apparatus

Gas chromatography analyses of alkyl esters were performed using a Carlo Erba MFC 500 (Carlo Erba, Milan, Italy) instrument equipped with a flame ionization detector (FID).

The FTIR spectra were acquired on a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), using a Rocksolid™ interferometer and a DigiTect™ detector system. The coupled attenuated total reflectance (ATR) accessory (Specac Inc., Woodstock, GA, USA) was equipped with a ZnSe 11 reflection crystal.

Materials, reagents and standards

The SPE cartridges (6 mL) STRATA Si-1 Silica (55 μm, 70 Å) packed with silica gel phase (1000 mg) were obtained from Phenomenex (Torrence, CA, USA). The silica gel stationary phase (60–200 mesh) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard used for FAAEs quantification [heptadecanoic acid methyl ester (C17:0ME)] was acquired from Sigma-Aldrich, as were methyl pentadecanoate, ethyl pentadecanoate, methyl palmitate, ethyl palmitate, methyl oleate, ethyl oleate, methyl linoleate and ethyl linoleate, which were used to identify the alkyl esters. All solvents used were analytical grade (Merck & Co. Inc., Darmstadt, Germany).

Determination of FAMEs and FAEEs by gas chromatographic analyses

Methyl esters of fatty acids and FAEEs were extracted from oil samples by three different methods (Bendini *et al.*, 2009b; Cerretani *et al.*, 2011; EC Reg. 61/2011) and quantified by gas chromatographic analyses following the analytical procedure reported in EC Reg. 61/2011. For standardising and harmonisation of the results, FAAEs were referred to the same analytical standard (C17:0 ME) for all samples analysed; moreover, all the response factors related to the GC-FID were set to 1.000.

Extraction of the alkyl esters by traditional liquid chromatography (samples 1–46)

This extraction follows the method reported in COI/T.20/DOC. NO. 28 (2009) 'Determination Of The Content Of Waxes, Fatty Acid Methyl Esters And Fatty Acid Ethyl Esters By Capillary Gas Chromatography' and recently adopted as official law by the European community (EC Reg. 61/2011 and corrigendum). A 0.5 ± 0.0001 g of the sample was mixed with 0.250 mL of standard solution of the internal standard (methyl heptadecanoate, C17:0 ME, 0.02% m/v). Next, 15 g of silica gel were suspended in *n*-hexane and settled spontaneously into a glass column for LC (internal diameter 15 mm, length 30–40 cm). The settling was complete with the aid of an electric shaker to make the chromatographic bed more homogeneous. Then, 30 mL of *n*-hexane were percolated to remove any impurities. The samples were transferred to the chromatography column with the aid of two 2-mL portions of *n*-hexane. The solvent was allowed to flow to 1 mm above the upper level of the absorbent. The alkyl esters were then collected eluting 220 mL of a freshly prepared mixture of *n*-hexane/ethyl ether (99:1, v/v) at a flow of about fifteen drops every 10 s. The resultant fraction was evaporated in a rotary evaporator until the solvent was almost removed, drying the last 2 mL under a weak flow of nitrogen. The fraction containing the methyl and ethyl esters was diluted with 2 mL of *n*-heptane, and 1 μL of this solution was injected.

Extraction of the alkyl esters by SPE (SPE 1) (samples 47–75)

For these twenty-nine samples, the extraction method followed the conditions described by Bendini *et al.* (2009b). A 0.2 ± 0.0001 g of oil sample was mixed with 250 μL of standard solutions of C15:0 EE and C17:0ME (both 50 μg g^{-1}), respectively, for the quantification of ethyl esters and methyl esters, and *n*-hexane was added to obtain a volume of 2 mL. This oil solution was split in two fractions of 1 mL and eluted separately. Silica SPE cartridges (1000 mg) were placed in an automatic vacuum elution apparatus and conditioned by passing 12 mL of *n*-hexane. Next, 1 mL of the oil solution was charged, and the solvent was pulled through at 0.5 mL min^{-1}, leaving the samples and the standards on the cartridge. The elution was made with 7 mL of the solvent mixture *n*-hexane:toluene (85:15, v/v), and this fraction was rejected. Next, the alkyl esters were collected by elution with 10 mL of the same mixture at a flow rate of 1 mL min^{-1}. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dry. The residue was dissolved in 200 μL of heptane, and a 1 μL of this solution was injected.

Extraction of the alkyl esters by SPE, with a different method (SPE2) (samples 76–81)

For these six samples, the extraction method followed the conditions described by Cerretani *et al.* (2011). A 1 ± 0.0001 g of oil sample was mixed with 500 μL of standard solutions of C17:0 ME, (200 μg g^{-1}) and 500 μL of standard solutions of lauryl arachidate (400 μg g^{-1}). Next, *n*-hexane was added to reach the volume of 5 mL. Silica SPE cartridges (1000 mg) were placed in an automatic vacuum elution apparatus and conditioned by passing 8 mL of toluene. Then, 0.5 mL of the oil solution was charged, and the solvent was pulled through at 0.5 mL min^{-1}, leaving the samples and the standards on the cartridge. The elution was made with 4 mL of the solvent mixture *n*-hexane/toluene (85:15, v/v), and this fraction was rejected. The alkyl esters were then collected by elution with 13 mL of the same mixture at a flow rate of 1 mL min^{-1}. The eluate was evaporated in a rotary evaporator at room temperature under vacuum until dry. The residue was dissolved with 200 μL of *n*-heptane, and 1 μL of this solution was injected.

GC condition of the analyses, followed for both extractions, by SPE and by traditional liquid chromatography

Compared to the official method, a slight modification in the programmed temperature of the oven was introduced to not exceed the maximum temperature of the capillary column. The official method employed a cold injector for direct on-column injection, while we set the temperature of the injector at 325 °C, with a split ratio fixed at 1:30. The capillary column was a ZB–5MS (Phenomenex) (30 m length × 0.25 mm i.d. × 0.25-μm-film thickness). Helium, at a flow rate of 1.2 mL min^{-1}, was the carrier gas. The oven temperature was programmed from 80 °C (kept for 1 min) to 140 °C at a rate of 15 °C min^{-1}, then raised to 325 °C at a rate of 4.5 °C min^{-1} and kept for 20 min. The FID detector was set at 325 °C. The amount of alkyl esters was expressed as mg of C17:0 ME kg^{-1} of oil. The average, for each sample, was calculated from three replicates.

Acquisition of FTIR spectra

Analyses were carried out in triplicate at room temperature. Spectra were acquired (thirty-two scans/sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 (Bruker Optics) software. The absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Each sample was uniformly spread throughout the crystal surface. Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone.

Data analysis

Data were exported in an ASCII compatible OPUS 6.0 software format (using a OPUS MACRO). PLS models were computed on respective training set samples for each parameter. The calculations were executed by MVC1 routines (Olivieri *et al.*, 2004) written for Matlab (Mathworks Inc., Natick, MA, USA). The moving window of variable size strategy (Ferraro *et al.*, 2001) was also implemented using MVC1. For each parameter, samples were treated independently. The selection of samples for calibration and validation groups was made using the Kennard & Stone algorithm, in both cases.

Results

PLS models for determination of FAMEs and FAEEs

As previously stated, the determination of the Σ (FAMEs + FAEEs) was carried out by previous extraction using three different pre-treatments, namely traditional LC (samples 1–46) and extraction by SPE with two different methods (SPE1, samples 47–75 and SPE2, samples 76–81). Table 1 shows the three groups of samples and their concentrations for Σ (FAMEs + FAEEs) and RFF. To develop and validate a robust PLS, only samples analysed by the official extraction method (EC Reg 61/2011) (LC) were taken into account. The obtained PLS parameters were applied on other groups of samples (SPE1 and SPE2). The content of Σ (FAMEs + FAEEs) for the first group EVOOs, determined by capillary GC, was in the range 13–116 (mg kg^{-1}): seven of the forty-six samples were close to the limits fixed for EVOOs. Figure 1a shows the full IR spectral range 4000–650 cm^{-1} for EVOO

Table 1 Sample groups and related contents for Σ (FAMEs + FAEEs) and RFF

	LC samples	SPE1 samples	SPE2 samples
Number of samples	46	29	6
Mean of Σ (FAMEs + FAEEs) content (mg kg^{-1})	47.1	54.0	437.5
Σ (FAMEs + FAEEs) range (mg kg^{-1})	13–116	9–159	32–749
Σ (FAMEs + FAEEs) SD (mg kg^{-1})	24	41	286
Mean of RFF	1.1	1.4	3.0
RFF range	0.4–1.8	0.3–4.1	1.9–4.1
RFF SD	0.40	1.0	1.1

FAEEs, ethyl esters of fatty acids; FAMEs, methyl esters of fatty acid; LC, liquid chromatography; RFF, ratio of FAEEs/FAMEs; SPE, solid phase extraction.

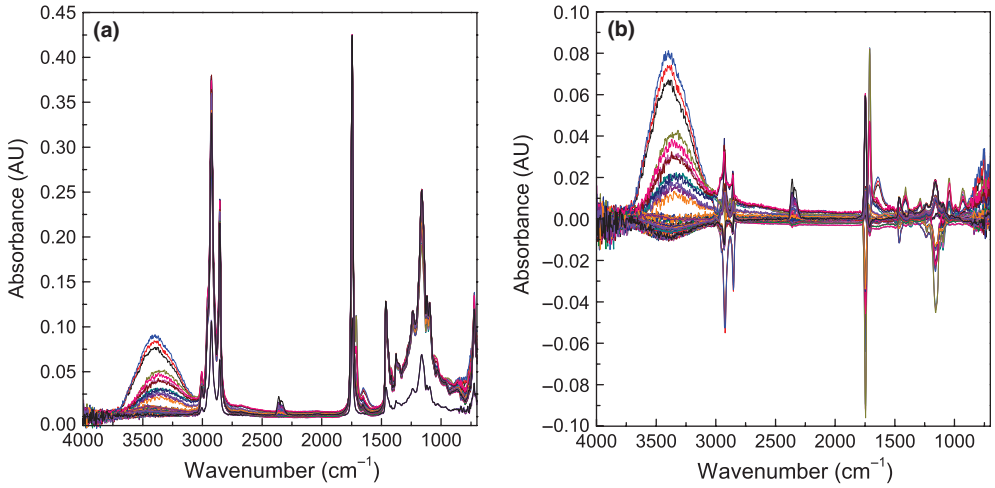

Figure 1 Full spectral range of Fourier transform mid-infrared (FTIR) for extra virgin olive oil (EVOOs) samples (a). Multiplicative Scatter Correction (MSC) and MC pre-treated FTIR spectra of EVOO samples (b).

samples. Different PLS calibration models were initially built by employing full spectra and reduced spectral ranges, obtained by a moving window of variable size strategy. However, none of these models provided an acceptable calibration, and predictions were unsatisfactory. Next, data were Mean-Centred (MC) and Multiplicative Scatter Correction (MSC) was employed to improve the performance of the method during calibration (pre-treated spectra are shown in Fig. 1b). Additionally, the spectral range was shortened to 2839.0–912.3 cm^{-1} to leave out regions where signal-to-noise ratio was very poor. By applying the Haaland and Thomas statistical criterion ($\alpha = 0.75$), the appropriate number of model dimensions was 6 (Haaland & Thomas, 1988). The results regarding calibration models between reduced pre-treated spectra and Σ (FAMEs + FAEEs) contents are reported in Table 2.

Table 2 Method parameters, statistical summary and figures of merits for Σ (FAMEs + FAEEs) PLS models

	LC	SPE1
Method parameters		
PLS factors	6	
Pre-treatment	MC – MSC	
Spectral range (cm^{-1})	2839.0–912.3	
Statistical summary: calibration		
Root Mean Square Deviation (RMSD, mg kg^{-1})	2.60	9.91
Percentage Relative Error In Calibration (REC, %)	5.98	17.35
R^2	0.98	0.95
Statistical summary: validation		
Mean recovery (%)	123	94
Relative Standard Deviation (RSD, %)	27	26
Figures of merit		
Sensitivity	0.00010	0.000026
Analytical sensitivity	0.36	0.19
Minimum detectable difference of concentration		
Σ (FAMEs + FAEEs), mg kg^{-1}	2.78	5.26
LOD Σ (FAMEs + FAEEs), mg kg^{-1}	8.8	7.0
LOQ Σ (FAMEs + FAEEs), mg kg^{-1}	29.3	20.9
Mean spectral residue (AU)	0.00029	0.000080

FAEEs, ethyl esters of fatty acids; FAMEs, methyl esters of fatty acid; LC, liquid chromatography; LOQ, Limit of Quantification; MSC, Multiplicative Scatter Correction; PLS, Partial Least Square; SPE, solid phase extraction.

PLS models for RFF determination

Following the same line of the previous developed models to estimate Σ (FAMEs + FAEEs), a PLS model, using LC samples, was built for detecting RFF. As in the PLS for Σ (FAMEs + FAEEs), MSC and MC spectral data pre-treatments were employed (Fig. 1b) to improve the performance of the model. Table 3 lists the calibration and prediction parameters used. Subsequently, the parameters of the calibration model obtained with LC samples were applied to SPE1 and SPE2 samples. As observed, the PLS model for RFF yields very good correlation coefficients and low Root Mean Square Deviation (RMSD) values; predictions were also very satisfactory, in terms of Relative Error in Calibration (REC) and recovery rate values (Table 3). The validation of the model was also carried out using the independent LC-validation mentioned above.

Table 3 Method parameters, statistical summary and figures of merits for RFF-PLS models

	LC	SPE1
Method parameters		
PLS factors	9	
Pre-treatment	MC – MSC	
Spectral Range (cm^{-1})	2839.0–912.3	
Statistical summary: Calibration		
Root Mean Square Deviation (RMSD, %)	0.15	0.17
Percentage Relative Error in Calibration (REC, %)	14	12
R^2	0.83	0.97
Statistical summary: validation		
Mean recovery (%)	103	107
Relative Standard Deviation (RSD, %)	17	36
Figures of merit		
Sensitivity	0.01	0.00
Analytical sensitivity	20.00	8.80
Minimum detectable difference of concentration	0.05	0.11

LC, liquid chromatography; MSC, Multiplicative Scatter Correction; PLS, Partial Least Square; RFF, ratio of FAEEs/FAMEs; SPE, solid phase extraction.

Discussion

PLS models for determination of FAMEs and FAEEs

The suitability of the proposed method for the objective was evaluated by analysing the model figures of merit and the results of validation samples. The values obtained for both RMSD and REC% were acceptable (Table 2). The Limit of Quantification (LOQ) was low enough considering the limits set by the EU for EVOO. Calibration R^2, which describes the goodness-of-fit of the predicted concentrations to their actual values was 0.98. The validation set exhibited almost quantitative recoveries that contain 100% in its confidence range (123 ± 27%). A Relative Standard Deviation (RSD) value of 27% shows the natural dispersion of the LC extraction method. The results (Fig. 2a) show good agreement between predicted and actual values on calibration and validation data sets. The slopes and intercepts of the curves depicted in this plot were close to unity and zero, respectively, indicating low bias and absence of systematic regression errors.

To demonstrate the inter-changeability and the transferability of the method (possibility of transference to another laboratory), a calibration model using a set of SPE1 samples and previously obtained PLS parameters (pre-treatments, PLS factors and spectral range obtained for PLS-LC model) was built, with satisfactory predictions. Almost quantitative recoveries (mean recovery = 94.38%, Table 2) were found, and high sample dispersion could be attributed to extraction procedure (Table 2 and Fig. 1b). The slope and intercept of the curves were also within required limits

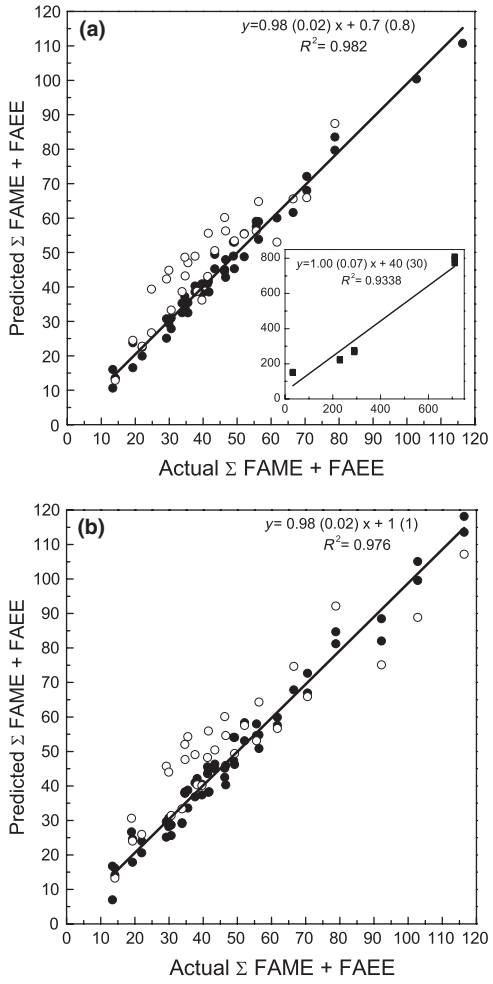

Figure 2 Actual vs. predicted Σ (FAMEs + FAEEs) for liquid chromatography (LC) calibration (●) and validation samples (○) (a). Prediction for SPE2 samples (a, subplot). Actual vs predicted Σ (FAMEs + FAEEs) content for SPE1 calibration (●) and validation samples (○) (b). Equations curves: Predicted = slope (SD_{slope})* Actual + intercept ($SD_{intercept}$). FAMEs, methyl esters of fatty acid; SPE, solid phase extraction.

(1 and 0). On the other hand, very few samples were analysed using the SPE2 method, making the development of a PLS model impossible. Nevertheless, SPE2 samples were analysed using PLS model developed with LC samples obtaining only an estimative prediction (Fig. 2a). As expected, almost all samples in the SPE2 group were out of the calibration range, making both figures of merit and statistical analyses meaningless.

PLS models for RFF determination

Precision and accuracy of RFF-PLS models were accessed by the evaluation of prediction errors of

(17%) and mean recovery (103%, mean value of sample recovery rates), and the latter were close to quantitative. Low bias and absence of systematic errors were demonstrated by the slopes and intercepts of the actual vs. predicted regression lines (Fig. 3a), which had values of unity and zero in their 90% joint confidence interval, respectively.

The same PLS parameters (pre-treatments, PLS factors and spectral range) were used to construct PLS models with SPE1 samples, and satisfactory results were found (Fig. 3b), which demonstrate the interchangeability of the method. Recoveries were similar to that obtained for LC samples (107 ± 37%, Table 2), but dispersion was higher. Consistent with the above, RFF values for SPE2 samples were out of the calibration range, being inappropriate to carry out predictions about this group. In addition, this group was too small to make a division in the validation and calibration groups: for this reason, SPE2 samples were not analysed.

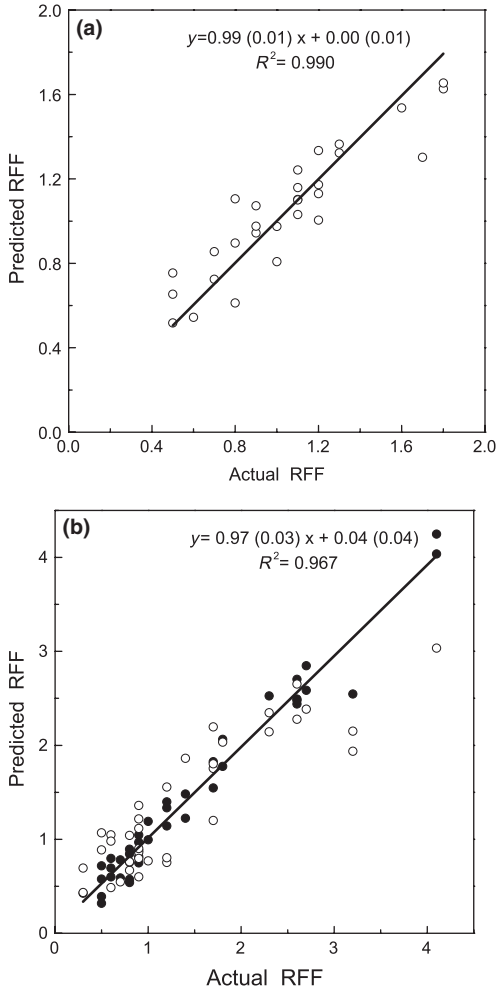

Figure 3 Actual vs predicted ratio of FAEEs/FAMEs (RFF) for liquid chromatography (LC) calibration (●) and validation samples (○) (a). Actual vs Predicted RFF content for solid phase extraction (SPE)1 calibration (●) and validation samples (○) (b). Equations curves: Predicted = slope (SD_{slope})* Actual + intercept ($SD_{intercept}$).

Conclusions

Fourier transform mid-infrared-PLS methodology was developed and demonstrated to be useful for analytical predictions of the Σ (FAMEs + FAEEs) content and RFF in EVOOs. The FTIR-PLS models provided results that were statistically similar to official procedures (LC), in terms of analytical performance, and are thus a useful tool for screening procedures. Moreover, the procedure permits high sample throughput, with significant time-saving, and is more environmentally friendly because no pre-treatment of samples was required. The results obtained here need to be confirmed through the acquisition of a larger set of olive oils, in terms of sample number and Σ (FAMEs + FAEEs) content, to increase the robustness of the model.

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ARTICOLO 13

Gómez-Caravaca, A.M., Maggio, R.M., Verardo, V., Cichelli, A., **Cerretani, L.** Fourier transform infrared spectroscopy-Partial Least Squares (FTIR-PLS) coupled procedure application for the evaluation of fly attack on olive oil quality. *LWT - Food Science and Technology* 50 (1), pp. 153-159, 2013 (IF 2,545).

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Fourier transform infrared spectroscopy–Partial Least Squares (FTIR–PLS) coupled procedure application for the evaluation of fly attack on olive oil quality

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ABSTRACT

A Fourier transform infrared spectroscopy–Partial Least Squares (FTIR–PLS) strategy for the determination of the quality of olive fruits and the respective virgin olive oil (VOO) has been developed. This methodology has been demonstrated as able to correlate the level of fly attack in olive oils with their FTIR spectra. A multivariate calibration model was built by the PLS algorithm using the 4000–700 cm^{-1} spectral range on pretreated data and an evaluation of some of the usual quality parameters of VOO (free acidity, fatty acids composition, oxidative stability by OSI time and phenolic compounds obtained by capillary electrophoresis) and was performed to corroborate the real influence of fly attack on the quality of the oils. Furthermore, the evaluation of the FTIR data showed some differences in the regions of the fatty acids and phenolic compounds depending on the percentage of fly attack on the olives. This non-destructive method easily allows a non-destructive measure and, at the same time, establishes the level of *Bactrocera oleae* attack in olive fruit thus analysing directly the olive oil obtained without any previous treatment.

1. Introduction

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean area; covering more than 8 million ha, accounting for almost 98% of the world crop which demonstrates the great economic and social importance of this crop. Virgin olive oil (VOO), as it is widely known, is obtained only by mechanical or physical procedures (Commission Regulation 1513/01) and because of this, its quality reflects the health status of the olives from which it was extracted. In fact, the quality of the VOO depends on its chemical composition that changes qualitatively and quantitatively in relation to a number of parameters including the health status of the fruits (Gallina-Toschi, Cerretani, Bendini, Bonoli-Carbognin, & Lercker, 2005). The action of parasites before harvest or fungal attack between harvesting and extraction are the main external agents responsible for unwanted metabolic processes in olives that lead to subsequent reduction in oil quality (Kiritsakis, 1998).

Bactrocera oleae or olive fruit fly is one of the most detrimental enemies of the quality of olive oil and it has been considered the most devastating insect pest in the Mediterranean region for 2000 years (Mraicha et al., 2010). There is evidence in literature about the reduction of oil yield, the increment of the acidity, the decrease of oxidative stability, the negative alteration of chemical composition and the changes in the flavour characteristics as a consequence of microorganisms' activity (Angerosa, Di Giacinto, & Solinas, 1992; Bendini, Cerretani, Cichelli, & Lercker, 2008; Gómez-Caravaca et al., 2008; Tamendjari, Angerosa, Mettouchi, & Bellal, 2009). For this reason, upon arrival at the mill, an estimation of the fly attack on olive fruit is conducted to assess the economic value of the olive fruits.

The severity of the negative effects depends on different variables such as the stage of the development of the olive fly, the intensity of the attack and the olive variety.

In the last few years, Fourier transform infrared (FTIR) spectroscopy has been used for the study of edible oils and fats. FTIR methods have demonstrated themselves to be rapid and non-destructive powerful analytical tools and in most cases they require minimal or no sample preparation. FTIR is also an excellent tool for quantitative analysis, since the intensities of the spectral bands are proportional to concentration. Several applications have

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been performed using this analytical approach together with chemometric methods: to detect olive oil adulteration (De la Mata et al., 2012; Lerma-García, Simó-Alfonso, Bendini, & Cerretani, 2011; Maggio, Cerretani, Chiavaro, Kaufman, & Bendini, 2010; Ozen & Mauer, 2002), to evaluate olive oil freshness (Sinelli, Cosio, Gigliotti, & Casiraghi, 2007), to assess oil oxidation (Guillén & Cabo, 2002; Muik, Lendl, Molina-Diaz, Valcarcel, & Ayora-Canada, 2007; Vlachos et al., 2006) and to study thermal stress (Maggio et al., 2011).

Thus, the aim of this research has been to evaluate the percentage of olive fly attack by a rapid FTIR methodology. Besides, other chemical parameters that have been described as directly related to fly attack, such as fatty acids composition and oxidative stability; free acidity and phenolic compounds have also been analysed.

2. Materials and methods

2.1. Samples

Thirty-two virgin olive oils from the Abruzzo region (Italy) that differed in the percentage of fly attack, variety of olive cultivars, technological system used (pressure or centrifugation, with or without a destoning phase) and produced during the 2009–2010 season were studied. The degree of infestation was calculated as the number of damaged olives per 100 fruits, considering both the presence of exit-holes and grubs (See Table 1).

2.2. Free acidity

Free acidity was determined according to the official method described in European Regulation EEC 2568/91 and amendments (Commission Regulation 2568/91).

Table 1
Variety and percentage of fly attack of the different olive oils studied.

% fly attack	Olive varieties
Outliers	
2.5%	Intosso
5%	Dritta
5%	Dritta
5%	Dritta
5%	Gentile
5%	Dritta, Leccino
7.5%	Mix
10%	Gentile
35%	Mix
60%	Gentile, Leccino
60%	Mix
Calibration	
2%	Dritta, Leccino
2.5%	Dritta, Intosso
2.5%	Leccino
5%	Leccino
5%	Gentile
7.5%	Tortiglione
7.5%	Leccino
15%	Gentile
15%	Intosso
25%	Mix
25%	Dritta
30%	Leccino
35%	Dritta
35%	Mix
45%	Mix
Validation	
4%	Dritta, Leccino
5%	Dritta
5%	Carpinetina
10%	Mix
10%	Mix
85%	Mix

Table 2
Fatty acid composition, free acidity at zero time (FA) and before 3 months of shelf life (FA3) and oxidative stability of virgin olive oils.

Samples	% fly attack	C 16:0	C 16:1	C 17:0	C 17:1	C 18:0	C 18:1	C 18:2	C 20:0	C 18:3	C 20:1	C 22:0	C 24:0	FA	FA 3	OSI
VOO1	2	12.80	1.00	0.05	0.08	2.70	74.60	7.40	0.40	0.60	0.20	0.10	0.07	0.43	0.38	27.90
VOO2	2.5	13.00	0.90	0.06	0.10	2.30	75.30	7.00	0.30	0.70	0.20	0.10	0.04	0.23	0.23	41.01
VOO3	2.5	13.00	0.90	0.06	0.20	2.20	74.50	7.70	0.30	0.70	0.30	0.10	0.04	0.20	0.23	35.03
VOO4	2.5	13.20	1.10	0.06	0.10	1.90	76.80	5.60	0.30	0.60	0.20	0.10	0.04	0.24	0.23	41.05
VOO5	4	13.00	0.90	0.04	0.06	2.70	74.90	7.00	0.40	0.60	0.20	0.10	0.10	0.37	0.37	27.94
VOO6	5	13.20	1.00	0.05	0.07	2.70	74.10	7.40	0.40	0.60	0.30	0.10	0.08	0.44	0.37	23.00
VOO7	5	12.80	1.00	0.05	0.08	2.70	75.00	7.00	0.40	0.60	0.20	0.10	0.07	0.52	0.42	24.15
VOO8	5	12.80	1.00	0.04	0.06	2.70	75.20	6.60	0.40	0.60	0.30	0.10	0.20	0.57	0.46	17.64
VOO9	5	13.00	1.00	0.06	0.07	2.60	74.90	7.00	0.40	0.60	0.20	0.10	0.07	0.64	0.52	18.01
VOO10	5	12.80	1.20	0.07	0.20	2.10	75.10	7.30	0.30	0.60	0.20	0.10	0.03	0.36	0.34	28.84
VOO11	5	14.40	1.30	0.06	0.10	1.80	74.60	6.40	0.30	0.70	0.20	0.10	0.04	0.28	0.25	24.11
VOO12	5	16.40	1.30	0.10	0.20	2.30	64.10	14.20	0.35	0.70	0.20	0.10	0.05	0.35	0.31	14.35
VOO13	5	16.00	1.30	0.10	0.20	2.30	65.20	13.60	0.26	0.70	0.20	0.10	0.04	0.53	0.46	13.51
VOO14	5	14.00	1.10	0.06	0.10	1.80	74.50	7.00	0.30	0.70	0.30	0.10	0.04	0.71	0.62	19.05
VOO15	7.5	14.20	1.30	0.06	0.10	1.80	75.90	5.30	0.30	0.70	0.20	0.10	0.04	0.26	0.30	37.59
VOO16	7.5	13.60	1.00	0.07	0.20	2.30	72.00	9.40	0.40	0.70	0.20	0.10	0.03	0.40	0.37	21.45
VOO17	7.5	16.80	1.40	0.10	0.20	2.40	62.60	15.20	0.36	0.60	0.20	0.10	0.04	0.26	0.25	21.04
VOO18	10	14.00	1.10	0.06	0.10	1.80	75.60	6.20	0.20	0.60	0.20	0.10	0.04	0.18	0.22	26.55
VOO19	10	14.00	1.30	0.06	0.10	1.90	75.30	6.10	0.30	0.60	0.20	0.10	0.04	0.58	0.52	19.89
VOO20	10	13.50	1.10	0.05	0.10	2.00	72.80	9.00	0.30	0.70	0.30	0.10	0.05	0.30	0.30	22.33
VOO21	15	16.00	1.30	0.10	0.20	2.20	64.40	14.50	0.30	0.70	0.20	0.06	0.04	0.57	0.49	9.84
VOO22	15	13.50	0.90	0.10	0.20	2.80	74.30	6.50	0.40	0.80	0.30	0.10	0.10	0.85	0.77	23.04
VOO23	25	12.80	1.20	0.07	0.10	2.00	76.60	6.00	0.30	0.60	0.20	0.10	0.03	0.30	0.28	27.67
VOO24	25	14.30	1.20	0.06	0.10	2.40	74.20	6.50	0.30	0.60	0.20	0.10	0.04	0.72	0.66	32.14
VOO25	30	14.40	1.40	0.03	0.10	1.80	74.50	6.50	0.30	0.70	0.20	0.05	0.02	0.93	0.75	18.04
VOO26	35	13.90	1.40	0.06	0.20	1.80	75.40	6.00	0.30	0.60	0.20	0.10	0.04	0.91	0.82	17.54
VOO27	35	13.80	1.10	0.05	0.10	2.00	74.70	6.80	0.30	0.70	0.30	0.10	0.05	1.12	0.83	13.12
VOO28	35	14.00	1.30	0.06	0.10	1.90	75.10	6.10	0.30	0.70	0.30	0.10	0.04	0.67	0.52	26.06
VOO29	45	14.20	1.20	0.07	0.30	2.20	73.00	7.70	0.30	0.70	0.20	0.10	0.03	0.50	0.48	20.02
VOO30	60	13.90	1.30	0.06	0.20	2.00	74.50	6.60	0.30	0.70	0.30	0.10	0.04	2.27	1.83	13.38
VOO31	60	16.70	1.50	0.10	0.20	2.20	64.60	13.20	0.36	0.80	0.20	0.10	0.04	3.78	3.01	8.01
VOO32	85	13.40	1.10	0.06	0.10	2.20	72.80	8.90	0.40	0.60	0.30	0.10	0.04	1.84	1.65	8.86

All fatty acids were expressed as g/100 g of oil, FA and FA3 were expressed as g of oleic acid/100 g of oil; OSI was expressed in hours.

2.3. Fatty acids determination

The fatty acid composition of oil samples was determined as methyl esters by capillary gas chromatography (GC) (Clarus 500 GC Perkin–Elmer Inc., Shelton, CT) analysis after alkaline treatment, according to Bendini, Cerretani, Vecchi, Carrasco-Pancorbo, and Lercker (2006). The alkaline treatment was carried out by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 mol l^{-1} potassium hydroxide in methanol according to Christie (1998).

2.4. Oxidation stability index (OSI) time

These analyses were carried out according to the method of Gómez-Caravaca et al. (2008). Briefly, the analyses were carried out in an eight-channel OSI instrument (Omnion, Decatur, IL, USA). Virgin olive oil samples (5.0 ± 0.1 g) were heated at 110 °C under atmospheric pressure, and air (150 mL min^{-1} of flow rate) was allowed to bubble through the oil. Under these conditions, the oxidative process reaches its final steps, and the short-chain volatile acids produced are recovered and measured conductimetrically in distilled water. The time required to produce a sudden increase in conductivity (due to volatile acid formation) determines an induction period (OSI time), expressed in hours and hundredths of hours, which can be used to measure the stability of oil.

2.5. Determination of polar phenolic fraction

To collect phenolic compounds from olive oil a liquid–liquid extraction method optimized by Pirisi, Cabras, Falqui Cao, Migliorini, and Mugelli (2000) was used. The dry extracts were dissolved in 0.5 mL of a methanol/water (50/50, v/v) solution and filtered through a 0.2 μm syringe filter (Whatman Inc., Clinton, NJ, USA). Extracts were frozen and stored at −43 °C.

A capillary zone electrophoresis method optimized by Carrasco-Pancorbo et al. (2006) was used to perform the capillary electrophoretic analyses. This method uses a capillary with 50 μm i.d. and a total length of 47 cm (40 cm to the detector) with a detection window of 100 × 200 μm, and a buffer solution containing 45 mmol l^{-1} sodium tetraborate pH 9.3. The capillary electrophoresis instrument was a P/ACE 5500 (Beckman Instruments, Inc., Fullerton, CA, USA) connected to a diode array detector.

2.6. FTIR analysis

All spectra were acquired using a Tensor 27™ FTIR spectrometer system (Bruker Optics, Milan, Italy), fitted with a Rocksolid™ interferometer and a DigiTect™ detector system coupled with an attenuated total reflectance (ATR) accessory. The ATR accessory (Specac Inc., Woodstock, GA, USA) was equipped with a ZnSe 11 reflection crystal. A small amount of the oil samples (about 1 g) was uniformly deposited on the crystal surface of ATR accessory and all analyses were carried out at room temperature. Spectra were acquired (32 scans/sample or background) in the range of 4000–700 cm^{-1} at a resolution of 4 cm^{-1}, using OPUS r. 6.0 (Bruker Optics) software. For each sample the absorbance spectrum was collected against a background obtained with a dry and empty ATR cell. Three spectra were recorded for each sample ($n = 3$). Before acquiring each spectrum, the ATR crystal was cleaned with a cellulose tissue soaked in *n*-hexane and then rinsed with acetone. Data was exported in ASCII compatible format of OPUS 6.0 software for further analysis.

2.7. Statistical analysis

PLS data analysis (Martens & Naes, 1989; Thomas, 1994), was performed in Matlab R2008a (Mathworks, Inc., Natwick, MA, USA) using MCV1 Toolbox written by Olivieri, Goicoechea, and Iñón (2004). PLS was run on mean-centred data. Statistical analyses and data plots were performed with OrigingPro 8 SR0 (OriginLab, Northampton, MA, USA).

The values reported are the averages of at least three repetitions ($n = 3$), unless otherwise stated. Tukey's honest significant difference (HSD) multiple comparison (oneway ANOVA) and Pearson's linear correlations are both at $p < 0.05$.

3. Results and discussion

3.1. Evaluation of tendencies among fly attacks and some quality parameters

An evaluation of some of the usual quality parameters of olive oil was performed to corroborate the real influence of fly attack on the quality of the oils.

The fatty acids detected in the olive oil samples analysed in this study are reported in Table 2. As expected, experiments revealed the oils tested contained large amounts of unsaturated fatty acids that were in the range of 64.4–78.2% of total fatty acids (Table 2). The most abundant fatty acid was the mono-unsaturated oleic acid (C18:1) that was determined in the range of 62.6–76.8%. Palmitic (C16:0) and linoleic acids (C18:2) were the second and third fatty acids ranging from 12.8 to 16.8% and from 5.3 to 15.2%, respectively. The oleic acid/linoleic acid ratio was higher than 7 except for five samples. This data was in agreement with literature (Gómez-Caravaca et al., 2008) and respected the characteristics established in the Commission Regulation 61/2011 for extra-virgin olive oils. No statistical differences ($p < 0.05$) were

Table 3
Phenolic content of olive oil samples quantified by capillary electrophoresis.

Samples	% fly attack	% simple phenols	% lignans	% secoiridoids	% EA	Total phenols mg/kg
VOO1	2	2.7	7.5	87.6	2.2	159.19 (c)
VOO2	2.5	0.7	4.3	93.4	1.7	286.06 (a)
VOO3	2.5	1.9	1.5	95.2	1.4	247.11 (a)
VOO4	2.5	2.3	4.3	91.3	2.2	200.96 (b)
VOO5	4	2.6	8.5	86.6	2.2	159.26 (c)
VOO6	5	4.4	10.2	83.7	1.7	120.41 (c,d)
VOO7	5	4.3	9.5	83.7	2.5	96.08 (d)
VOO8	5	2.7	12.3	82.9	2.1	87.29 (d)
VOO9	5	5.2	15.6	76.8	2.4	71.46 (d)
VOO10	5	3.0	4.1	92.9	0.0	154.43 (c)
VOO11	5	5.6	11.9	81.2	1.3	49.52 (f)
VOO12	5	3.5	28.1	64.9	3.5	100.14 (d)
VOO13	5	4.7	31.8	60.4	3.2	66.81 (e)
VOO14	5	6.8	22.6	70.7	0.0	36.42 (g)
VOO15	7.5	1.2	2.1	94.8	2.0	153.24 (c)
VOO16	7.5	4.8	13.5	80.6	1.1	140.87 (c)
VOO17	7.5	3.1	17.8	75.6	3.4	161.28 (c)
VOO18	10	4.4	3.1	91.2	1.3	85.71 (d)
VOO19	10	8.4	9.5	81.2	0.9	26.18 (h)
VOO20	10	3.0	5.8	91.2	0.0	116.32 (d)
VOO21	15	5.4	22.6	72.0	0.0	35.60 (g)
VOO22	15	1.4	8.1	89.0	1.5	130.77 (c,d)
VOO23	25	4.0	6.8	87.3	1.8	100.55 (d)
VOO24	25	3.1	5.6	89.7	1.6	169.26 (b,c)
VOO25	30	7.0	17.5	75.4	0.0	18.68 (i)
VOO26	35	4.9	12.0	83.0	0.0	20.92 (i)
VOO27	35	4.0	28.5	64.9	2.7	61.18 (e)
VOO28	35	5.6	7.1	86.2	1.1	49.09 (f)
VOO29	45	3.7	11.0	84.2	1.0	76.28 (e)
VOO30	60	12.7	8.3	78.9	0.1	15.36 (i)
VOO31	60	5.9	15.1	79.0	0.0	26.59 (h)
VOO32	85	5.2	19.7	75.1	0.0	16.24 (i)

Simple phenols, lignans and elenolic acid (EA) were quantified with a calibration curve of 3,4-dihydroxyphenilacetic acid at $\lambda = 214$ nm secoridoids were quantified with a calibration curve of oleuropein glucoside at $\lambda = 214$ nm.
Different letters in the same column means significantly different values ($p < 0.05$).

reported among single and total fatty acid composition and percentage of fly attack.

Furthermore, according to Gómez-Caravaca et al. (2008), *B. oleae* attack was correlated to free acidity ($r = 0.79$, $p < 0.05$) and free acidity after three months of oil storage ($r = 0.81$, $p < 0.05$). The evaluation after three months was performed because this period is long enough to observe the beginning of the oxidative reactions and to see differences in the free acidity.

The fly attack did not influence the oxidative stability determined by OSI (Table 2).

Table 3 shows the phenolic composition of the samples. It was observed that the phenolic content changed from 16.3 to 286.1 mg/kg of oil. As reported in literature (Bendini, Cerretani, et al., 2007), the secoiridoids were the most representative family of phenolic compounds (71–95.2%). Lignans were the second family with an average content of 9.4%; simple phenols were in third position representing 3.2% and elenolic acid (obtained by cleavage of secoiridoids) was in a concentration of 1.7% of total phenolic content. The lowest phenolic contents were found for olives with the highest influence of fly attack (Tamendjari et al., 2009). A decrease of secoiridoid derivatives and elenolic acid in cases of strong fly attack may be related to the increase of the polyphenol oxidase activity due to the entrance of oxygen from the exit hole made by fly larvae, which enhances phenolic oxidation (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Gómez-Caravaca et al., 2008).

The trend graphics between the different parameters analysed (free acidity, oleic/linoleic acid ratio, oxidative stability by OSI time and phenolic compounds obtained by capillary electrophoresis) and the percentage of fly attack were also defined (Fig. 1, parts A–D).

Moreover, the free acidity at initial time and after three months increased with fly attack following grade 2 polynomial behaviour (Fig. 1, parts A and B). This fact is in agreement with previous results found in bibliography (Mraicha et al., 2010; Pereira, Alves, Casal, & Oliveira, 2004) where free acidity has been described as one of the parameters that better correlates to fly infestation. Thus, it is possible to attribute this increase in acidity to hydrolytic processes occurring in damaged olives.

The analysis of the oxidative stability showed a lineal decrease at higher percentages of *B. oleae* attack (Fig. 1, part C). Despite this, some healthy oils presented low oxidative stability values because this parameter is affected by several factors (Aparicio, Roda, Albi, & Gutiérrez, 1999; Bonoli, Bendini, Cerretani, Lercker, & Gallina-Toschi, 2004). Finally the behaviour of the phenolic compounds was evaluated. The three main families of phenolic compounds of olive oil (simple phenols, lignans and secoiridoids) and total phenolic compounds had a similar tendency. An exponential decrease of each of them was observed when fly attack increased. Fig. 1 part D shows the graphic of total phenolic content vs. fly attack. These results are in agreement with numerous authors that have reported a high negative linear correlation between the infestation degree and total phenols content (Bendini, Gómez-Caravaca, et al., 2007; Koprivnjak et al., 2010).

3.2. Evaluation of FTIR data

FTIR olive oil spectra showed the typical absorption bands. Fig. 3A shows the untreated FTIR spectra of two oils from the olives with the lowest (continue line spectra) and the highest (dotted line

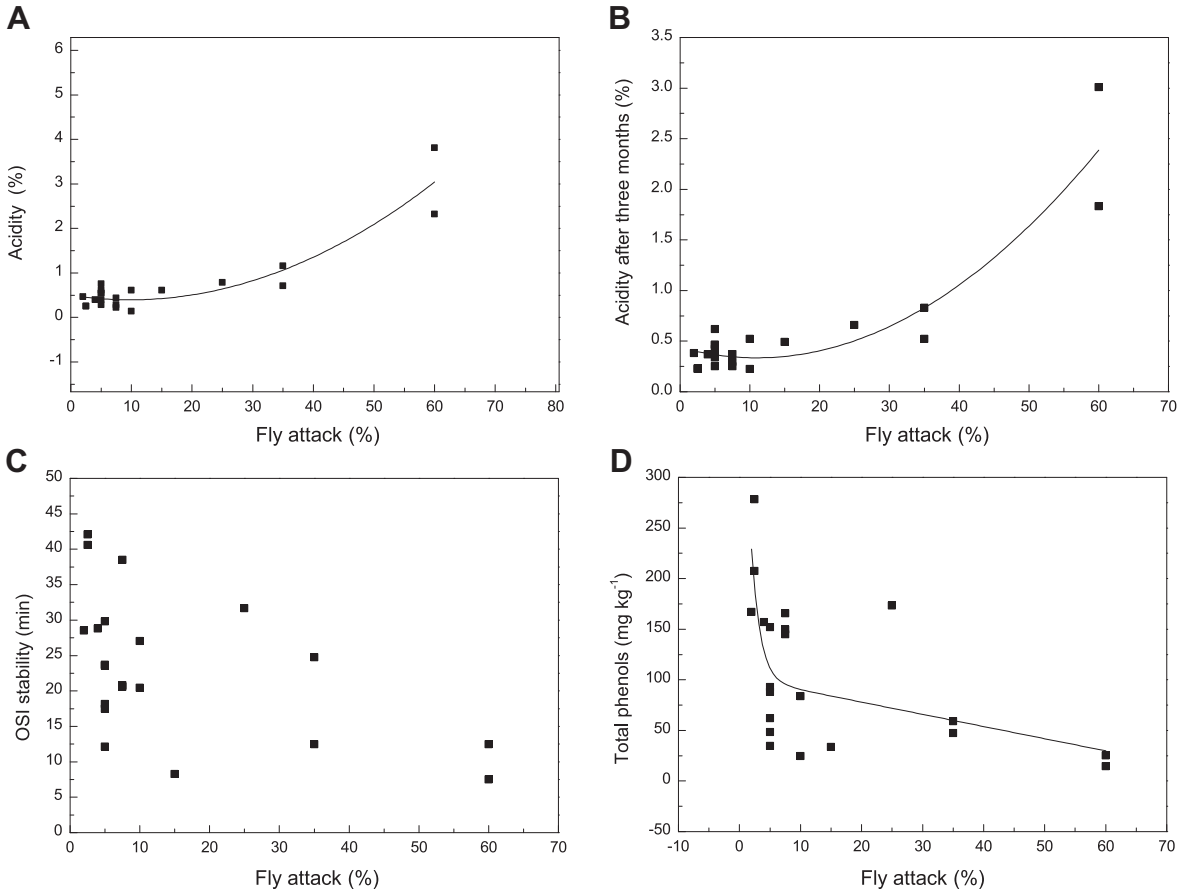

Fig. 1. Evaluations of tendencies among fly attack and quality parameters: A) Acidity, B) Acidity after three months, C) OSI, oxidative stability index and D) Total phenol content.

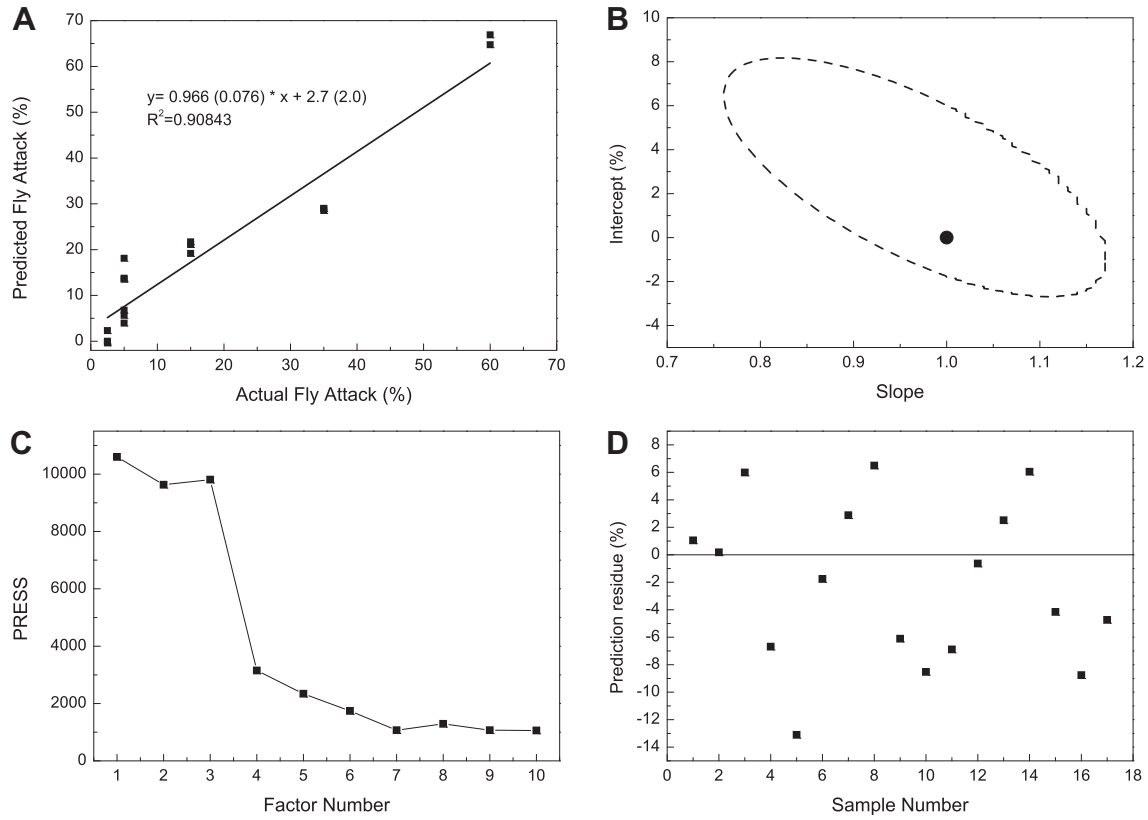

Fig. 2. Evaluation of validation set samples: A) Curve actual vs. predicted fly attack values, B) Joint confidence ellipse test, C) Cross-validation of calibration set, PRESS value vs. PLS factors, optimal number of factors 7, and D) Concentration residues plot.

spectra) percentages of fly attack. As the fly attack increases, several changes occurred in the FTIR spectra of the respective olive oils.

The main changes in the spectra appeared in the following regions:

a) 1800–1630 cm^{-1}; according to Beltrán Sanahuja, Prats Moya, Maestre Pérez, Grané Teruel, and Martín Carratala (2009), this region is related to the vibration of the carbonyl group of triacylglycerol esters. It is normally observed in the infrared spectrum of olive oil that both aliphatic and carbonyl groups give a very strong absorption in the corresponding regions. The more oxidized samples demonstrated a high absorption in this zone. Nunes, Martins, Barros, Galvis-Sánchez, and Delgadillo (2009) describe an indirect determination of olive acidity that allows a rapid evaluation of olive oil quality using FTIR methodology. As reported in the Fig. 3B a strong band was located at 1743 cm^{-1}. Moreover, the samples that reported a high FA (corresponding to a high fly attack) showed an absorbance at 1728 cm^{-1} which overlaps with the stretching vibration at 1746 cm^{-1}. According to Vlachos et al. (2006), the absorbance at 1728 cm^{-1} may be due to the production of saturated aldehyde functional groups or other secondary oxidation products. The sign of O–H deformation of water is evident near band 1640 cm^{-1} in the spectra, as illustrated in Fig. 3B. As mentioned by Hayati, Man, Tan, and Aini (2005), evaporation of water occurred in the oxidized samples, leading to a significant decrease in the absorbance of the band.

b) 1300–800 cm^{-1}; the wavelengths numbers of FTIR spectra at 827, 1039, 1115, 1143, 1286 cm^{-1} were assigned to C–H alkenes, –C–O alcohols, C–OH alcohols, –OH aromatic, C–O alcohols (Gorinstein et al., 2009). According to Bureau, Ścibisz, Le Bourvellec, and Renard (2012) the region 1225–950 cm^{-1} was assigned to aromatic C–H in plane bend.

Briefly, this region of fingerprinting (1330–800 cm^{-1}) was considered for the phenolic compounds estimation. Basically, the olive oils from highly fly attacked olives reported lower absorbance (Fig. 3C).

3.3. FTIR–PLS models for the fly attack

In order to correlate the level of fly attack in olive oils with their FTIR spectrum, a multivariate calibration model was built by the PLS algorithm (Fig. 2, parts A–D), using the full spectrum (4000–700 cm^{-1}). Mean centre and multiplicative scatter correction were used as data pretreatment. From the initial set of samples a calibration set and a validation set by random selection were performed (Table 1). Besides, a cross-validation process was carried out within the calibration set, using the "leave one out" procedure (Picard & Dennis Cook, 1984) in order to establish the PLS parameters. The appropriate number of PLS-factors were determined applying the criterion of Haaland and Thomas (1988) based on the minimal stable PRESS (Fig. 2, part C).

Table 4 reports the results regarding statistical summary and figures of merit of the model. Low values were obtained for RMSD (root mean square deviation, measures the average error in the analysis) and REC% (percentage relative error in calibration, evaluates the goodness of fit of the calibration data to the models), which measure the average error in the analysis and evaluate the goodness of fit of the calibration data to the models developed during calibration. Acceptables LODs (limit of detection) and R^2

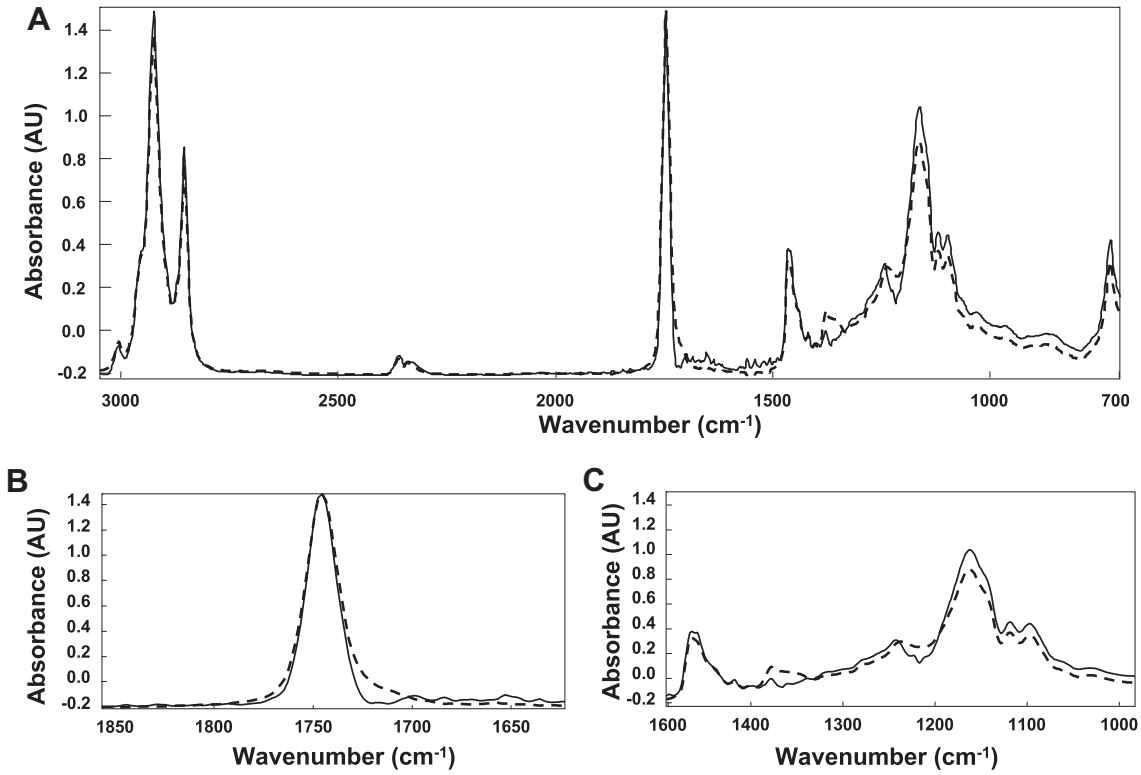

Fig. 3. Untreated FTIR spectra of two oils from olives with low (continue line spectra) and high (dotted line spectra) fly attack.

were also obtained. These later values were limited by the small number of actual samples that could be obtained.

3.4. Evaluation of validation samples

As stated above (Section 3.3. *FTIR–PLS models for the fly attack*), the validation set was constructed by random selection from initial set of samples (Table 1). In the Fig. 2, part A, the fitting curve of actual vs. predicted values (attack, %), shows a good correlation ($R^2 = 0.9084$). Additionally, the curve had a slope close to 1 and intercept close to 0 demonstrated by the joint confidence ellipse [containing the point (1, 0)]. This fact implies no bias or systematic errors may occur. Besides, prediction errors uniformly distributed (Fig. 2, part D) also validate the linearity of the model. Finally, during the evaluation of the validation set it was observed that the spectral residues were uniformly distributed, showing that the validation samples contain no unmodeled interference.

Thus, it can be concluded that the method can model the problem correctly and that FTIR represents an appropriate technique to quickly determine the extent of fly attack and to establish the healthy status of the olives. This fact is very useful because olive oils highly attacked by *B. oleae* are described to have worse quality characteristics and, therefore, in most cases extra virgin olive oils can not be obtained from these kinds of olives (Mraicha et al., 2010).

Table 4
Statistical summary and figures of merit of fly attack FTIR–PLS model.

Statistical summary		Figures of merit	
Factors	7	Sensitivity:	0.00058
RMSD[a]	6.06	Analytical sensitivity:	0.75
REC[a]	41.60	Selectivity	0.2
R^{2a}	0.9090	Mean spectral residue:	0.00069
		LOD = 3.3*SD	4.36

[a] RMSD $= [(\sum_{i=1}^{N}(y_i - \hat{y}_i)^2)/N]^{0.5}$, $R^2 = [\sum_{i=1}^{N}(y_i - \hat{y}_i)^2]/[\sum_{i=1}^{N}(y_i - \overline{y}_i)^2]$ and REC(%) = 100*RMSD/$\overline{y}$, where y, $\hat{y}$ and$\overline{y}$ represent the true, predicted and mean value of analyte, in the N training samples, respectively.

4. Conclusions

An ATR-FTIR–PLS based strategy for the determination of quality of olive fruits and consequently of VOO has been developed. This methodology is non-destructive and, at the same time, allows the level of *B. oleae* attack to be established by analysing directly the olive oil obtained. Therefore, this method could be very useful for the qualitative control of the raw material (olives) in the step prior to the production of olive oil. Besides, the FTIR instrument could be used together with a small laboratory mill (i.e. Abencor) and, in this way, the simultaneous determination of the quality of olive oil and the quality of the olives could be estimated.

It is also important to highlight that the principal advantages of the use of these instruments are that it would be possible to avoid consuming organic solvents and, besides, analysis could be performed even by non lab-qualified workers.

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