Comparison of methods for determining the fatty acid composition of photosynthetic tissues

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Abstract

The fatty acid (FA) composition of photosynthetic tissue differs from that in other plant or animal tissues. In leaves, the lipid fraction constitutes less than 10% of the dry weight and is mostly located in the chloroplasts. An extraction solvent should dissolve polar lipids readily, but should also overcome interactions between the lipids and the tissue matrix. A mixture of chloroform/methanol (C/M) is commonly used. However, less toxic alternative methods such as hexane/isopropanol (H/I) and ethanol (E) have been suggested. In this preliminary study we compared the effectiveness of these three methods which are used as standard extraction protocols for FA analysis of plant material at three different European Universities. C/M extraction gave the highest total FA content and H/I the lowest, suggesting that C/M is indeed the best general-purpose lipid extraction solvent. Significant differences were also observed for FA composition including the ratio of saturated to unsaturated FA indicating selectivity of the various solvents in extracting different individual FA. Further and more detailed investigations are required to confirm this hypothesis.

Introduction

Classically, determination of FA composition is done in three steps: first, lipids from the sample are extracted with a solvent; second, the isolated lipids are (trans)esterified to form fatty acid methyl esters (FAMEs); and in the last step, FAMEs are quantified by gas chromatography (GC). Various methods are used for these three steps; however, not all of them are suitable for plant material, as the lipid composition of photosynthetic tissue differs significantly from that of other sources. This lipid fraction constitutes less than 10% of the dry weight of leaves and mainly consists of complex polar and highly unsaturated membrane lipids. Consequently, a chosen solvent should not only be suitable to dissolve polar lipids readily, but should also overcome the interactions between the lipids and the tissue matrix.

The extractability of lipids from a given tissue is variable and depends both on the nature of the tissue and of the lipids (Christie, 2003). For instance, simple lipids or triacylglycerols are extracted relatively easily, while galactolipids or phospholipids are more difficult to extract since they are constituents of membranes. Furthermore, lipids lacking polar groups are very soluble in hydrocarbons such as hexane, toluene and cyclohexane, or in moderately polar solvents, such as chloroform. Lipids with polar groups are only slightly soluble in these solvents. The polar lipids are extractable from plant tissue only in highly polar solvents such as ethanol or methanol, which are able to counterbalance the hydrogen bonds and ionic forces between these membrane-associated lipids and proteins. A combination of moderately polar chloroform and polar methanol (2/1, v/v), C/M (Folch et al., 1957) has proven to extract both polar and non-polar lipids from various kinds of tissues more fully than most other solvents (Christie, 2003). However, since both components are harmful to the analysts and environment other methods have been applied over the years to find a solvent system with low toxicity, e.g. Hara and Radin

(1976) and Elgersma et al. (2003). Consequently, various methods are used for the extraction of FA from herbage tissues; however, there is no assurance that results obtained with these different methods are comparable with each other. Therefore, in this preliminary study we compared two extraction methods: hexane/isopropanol, H/I (Nourooz-Zadeh and Appelquist, 1988) and ethanol, E (Elgersma et al., 2003) for their efficiency in the extraction of lipids from photosynthetic tissue against the "reference" method, C/M (Folch et al., 1957).

Material and Methods

Plant material was collected from an experimental field at Wageningen University, the Netherlands. Fresh herbage (± 200g) was sampled immediately, collected in plastic bags in dry ice (-80˚C) and moved to the lab. Three perennial ryegrass samples with a different leaf blade proportion were chosen. In addition, a grass silage sample was taken from an experimental farm of Wageningen University. Each sample was divided into four sub-samples, which were kept frozen. A paper cutter was used to cut the sub-samples into 5-10 mm pieces, that were representatively divided into three centrifuge tubes, as individual replicates for each method. Tubes were transported frozen (-80°C) and analyzed on the same day (11 days after sampling) in each lab with their standard method: chloroform/methanol (2/1, v/v) (C/M, Folch et al., 1957), hexane/isopropanol (H/I, Nourooz-Zadeh and Appelquist, 1988) and ethanol (E, Elgersma, et al., 2003). The (trans)esterifications were done as described by: Raes et al. (2001), Sukhija and Palmquist, (1988), and Badings and de Jong (1983), for C/M, H/I and E, respectively. The GC conditions were similar for all methods, except differences in the length of the column, which was 100 m for C/M and H/I (CP-Sil-88 for FAME, Chrompack, The Netherlands), and 30 m for E (ZB-Wax, Zebron, Phenomenex, USA).

Statistical analysis

Data were processed using the SPSS statistical package (SPSS for Windows, Rel. 11.0.1, 2001. SPSS inc., Chicago, USA). Grass (two-way mixed model Anova) and silage (one-way Anova) samples were analyzed separately. Comparison of means for the individual treatments was done at the 5% significance level based on the *F*-test of the analysis of variance.

Data summary

The assessment of extraction solvent and methylation procedure on grass FA results was based on the seven major grass FAs (C14:0, C16:0; C16:1, C18:0; C18:1; C18:2 and C18:3), called BIG7. The profiles of these seven individual FAs were re-calculated relative to the total of BIG7 (93% (C/M and H/I) to 95% (E) as proportion of total FAs). Concentrations of total BIG 7 and individual FAs differed significantly between methods. Data for concentrations of three major FA (C16:0, C18:2 and C18:3 are presented in Figure 1. For grass samples, the highest concentrations were reported with the C/M method and the lowest with H/I, which gave only 8% of C/M value for BIG7.

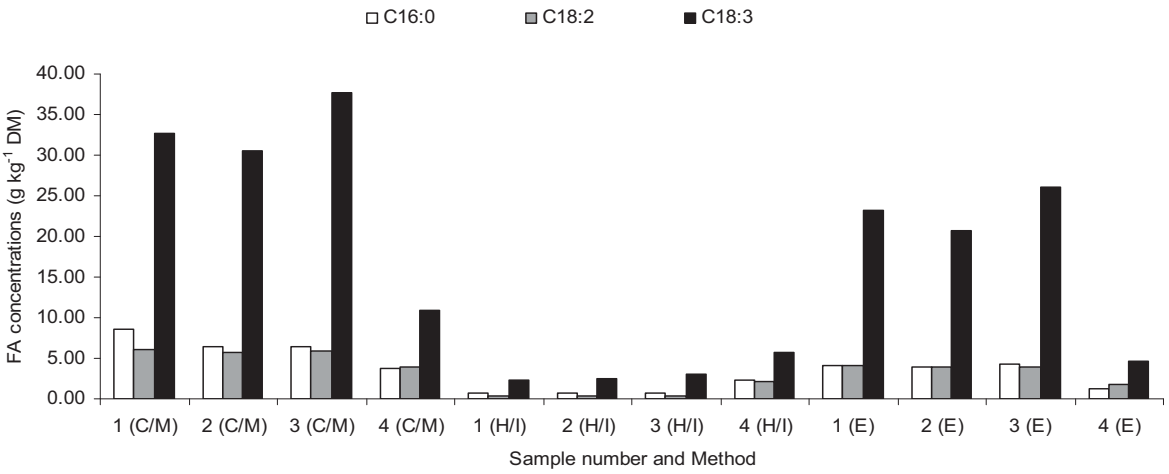

Figure 1. Effect of three extraction methods (C/M, E, H/I) on estimates of the concentrations of three major FAs in four grass samples (1-4)

Table 1. Effect of three extraction methods (C/M, E, H/I) on estimates of FA profiles and of their total FA concentration (BIG7) in four grass samples (1-4)

					(%)				(g kg^{-1} DM)
Method	Sample	C14:0	C16:0	C16:1	C18:0	18:1	18:2	18:3	BIG7
					FRESH GRASS SAMPLES (n=4)				
C/M	1	3.46	15.52	1.68	3.24	5.76	11.16	59.18	55.31
	2	4.17	13.23	2.09	2.00	3.24	11.92	63.35	48.12
	3	3.56	11.58	2.59	1.36	1.93	10.54	68.43	55.11
H/I	1	0.83	18.36	0.74	5.21	2.68	10.96	61.22	3.75
	2	0.77	17.38	0.50	5.30	2.74	10.91	62.39	3.90
	3	0.56	14.67	0.16	4.53	1.97	8.55	69.56	4.38
E	1	0.26	12.85	0.20	0.96	1.85	12.69	71.20	32.53
	2	0.26	13.12	0.29	1.10	2.44	13.04	69.76	29.78
	3	0.19	11.99	0.10	0.89	1.63	11.18	74.10	35.24
Method (M)	Sign	***	**	**	***	NS	**	**	***
Sample (S)	Sign	NS	NS	NS	NS	NS	**	*	NS
	s.e.d.	0.145	0.698	0.092	0.355	0.547	0.403	1.508	1.098
M * S	Sign	NS	NS	***	NS	NS	NS	NS	NS
					SILAGE SAMPLES (n=4)				
C/M	4	9.97	16.39	5.78	1.52	2.80	16.53	47.02	23.32
H/I	4	1.20	21.27	0.70	2.02	3.06	19.49	52.27	10.95
E	4	0.81	15.19	0.24	1.36	2.55	21.56	58.21	8.08
Method (M)	Sign	***	***	***	***	***	***	***	***
	s.e.d.	0.398	0.210	0.193	0.129	0.335	0.266	0.729	0.387

Asterisks indicate significant effects: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant.
s.e.d. standard error of difference.

Differences in estimates of the proportions of individual FAs were also significant between methods (Tab. 1). It was distinctive that with H/I the highest profiles of C16:0 and C18:0 were reported, with C/M the highest profiles of C14:0 and C16:1, and with E the highest profiles of C18:3. Moreover, the ratio between saturated and unsaturated FAs was significantly different for the investigated methods, with H/I the estimated ratio was highest (0.33, 0.31, 0.25 and 0.32 for sample 1, 2, 3 and 4, respectively) and with E lowest (0.16, 0.17, 0.15 and 0.21 for sample 1, 2, 3 and 4, respectively).

Discussion

In this preliminary research, no distinctive evaluation has been made of the extraction and esterification procedure and GC conditions. Consequently, differences in estimated values between methods could not be related to extraction or (trans)esterification separately, only by the overall procedure. This discussion is limited to the first two steps of the procedure, extraction and (trans)esterification, since the methods and equipment used for GC analysis of FAMEs were similar for the three methods.

Polar ethanol was presented as a successful solvent for lipids from the liver (Lucas and Ridout, 1970), cyanobacterium (Mendes et al., 2005 and 2006) and fresh grass and silage (Elgersma et al., 2003). In the present comparison, in agreement with Mendes et al. (2005 and 2006), E yielded 60 to 70% lower concentrations of total and individual FAs than C/M. Although H/I was presented previously to be a good solvent for the lipids from the brain tissue (Hara and Radin, 1976) and milk powder products (Nourooz-Zadeh and Appelquist, 1988), it seems to be unsuitable for fresh plant tissue with complex and polar membrane-associated lipids as only 8% of the lipids were estimated compared to C/M. This conclusion is consistent with the fact that hexane is a good solvent for lipids that do not contain markedly polar groups, for example triglycerides or cholesterol esters (Christie, 2003) and that isopropanol is used mainly to deactivate enzymes (Hawke, 1973). Overall, the mixture of chloroform/methanol once more proved to be a more general solvent, as it extracted lipids more readily than any other (combinations of) solvents. Furthermore, considering the significant differences in the FAs profiles and ratio of saturated to unsaturated FAs between these three methods, it is apparent that the used solvents showed selectivity in extracting different lipid classes. E appeared to be a good solvent for membrane-associated lipids, especially galactolipids containing high proportion of C18:3, whereas H/I does not have these properties and consequently extracted fewer lipids from the photosynthetic tissue.

Moreover, the (trans)esterification step seems to be especially crucial for explaining the differences in the concentration of total and individual FAs for the silage sample. Elgersma et al. (2003) found that the level of free fatty acids (FFA) in silage samples varied from 27 to 73% of total FAs. Acid-based methylations, used with the C/M and H/I methods, esterify FFAs or transesterify FAs linked by ester bonds (EFA) to glycerol or cholesterol (Christie, 2003). In contrast, alkaline-based methylation, used after E extraction, only transesterifies EFAs but does not esterify FFAs. So the total concentrations of FAs in silage samples analyzed with C/M and H/I were equal to FAMEs of FFAs plus EFAs, whereas in samples analyzed with E results represented only FAMEs of EFAs. Consequently, the differences in the total concentration of FAs especially between C/M and E might be explained not only by the extraction step, but also by the (trans)esterification step. On the other hand, since the proportion of FFAs in fresh samples is usually low, from 1 to 2% (Elgersma, et al. 2003; personal communication M. Lourenço), the differences in the total concentration of FA in fresh grass samples cannot be explained by the methylation step, and thus are mainly a result of the extraction.

Conclusions

Three methods showed significant differences in concentration and proportion of FAs in fresh grass and silage. These were mainly related to the selectivity of the used solvents in extracting different lipid classes. The mixture of chloroform/methanol (2/1, v/v) proved to be the most general and efficient solvent in extracting lipids. On the other hand, hexane/isopropanol appeared to be unsuitable for the extraction of membrane-bound polar lipids.

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