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Review

Chromatographic and non-chromatographic assay of L-carnitine family components

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CONTENTS

1. INTRODUCTION

The endogenous pool of L-carnitine includes L-carnitine (LC), short-chain L-carnitine esters [mainly acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC)] and medium- and long-chain L-carnitine esters. All these esters are equilibrated with L-carnitine through the L-carnitine acyl transferase catalysis, which possesses different specificities for each substrate, the acetyl-, octanoyl- and palmitoyltransferases (CAT, COT and CPT, respectively) being the most investigated [1–3]. L-Carnitine is used in the therapy of primary and secondary deficiency, and in various other diseases such as dislipoproteinemia.

When exogenously administered, ALC proved to achieve useful results in some disturbances in the central nervous system, whereas PLC has shown very interesting results in the cardiovascular field [4–7]. This paper aims to give a comprehensive review of all the methods used in the assay of LC, ALC, PLC and, in general, the physiological components in the L-carnitine pool, with specific reference to their intrinsic validation, mainly those of enantioselective methods employed in the pharmacokinetic field.

2. L-CARNITINE ASSAY METHODS

LC was isolated and known to be a constituent of animal tissues as early as 1905, but it was assayed only much later. After the discovery that LC is an essential nutrient for the yellow meal worm *Tenebrio molitor*, Fraenkel [8] and Friedman and Fraenkel [9] set up the first assay procedure in the early 1950s. This was based on the growth of the worm in the presence of the testing material, compared with the growth in the presence of a known amount of LC. However, this bioassay was not adequate to estimate LC in biological fluids and tissues, owing to lack of specificity, sensitivity and precision. Chemical methods were also developed, based on complex formation by carnitine's quaternary ammonium groups and chromophoric compounds, the most widely employed test being bromophenol blue [10,11]. But these colorimetric procedures did not overcome the specificity problems and have sensitivity limitations. The latter remained until the enzyme assay was developed, coupling a specific enzyme reaction with various detection techniques, the most sensitive being the radioenzyme procedure. The principal methods currently available are reported in detail in the following sections.

2.1. Spectrophotometric and fluorometric analysis

2.1.1. L-Carnitine

A significant step in LC measurement was the enzyme assay developed by Marquis and Fritz in 1964 [12]. They used the purified enzyme CAT, which catalyses the following stereospecific reaction between acetyl-coenzyme A (acetyl-S-CoA) and LC:

$$\text{acetyl-S-CoA} + \text{LC} \overset{\text{CAT}}{\rightleftharpoons} \text{ALC} + \text{CoASH}$$
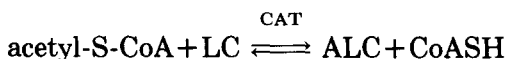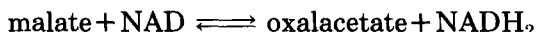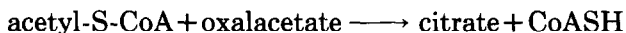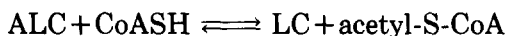
Only the L-isomer is active, the D-isomer being either inactive or, at high concentrations, inhibiting the enzyme.

The reaction produces a stoichiometric amount of CoASH, which is allowed to react with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), releasing the chromogenic thiophenolate ion, which absorbs at 412 nm. The method had better specificity and sensitivity (5 nmol) than previous ones and was used to determine the free LC content of rat tissues, although the perchloric acid extracts were heated at 90°C for 5 min at pH 8.5, in order to remove interferences from endogenous sulphydryl groups, which react with DTNB. A tissue blank is required in any case and, in these conditions, acetyl- and other short-chain acyl-L-carnitine are unstable. Other authors coupled the free CoASH formed from the above CAT reaction with other reactions, using several spectrophotometric or fluorimetric methods. Similar procedures have been proposed for short-chain LC esters [13–16]. Automated spectrophotometric methods have also been described [17,18].

2.1.2. Acetyl-L-carnitine

ALC has traditionally been determined by a spectrophotometric of fluorimetric method based on changes in reduced nicotinamide-adenine dinucleotide ($NADH_2$) concentration derived from the following coupled reactions:

$$\text{ALC} + \text{CoASH} \rightleftharpoons \text{LC} + \text{acetyl-S-CoA}$$

$$\text{acetyl-S-CoA} + \text{oxalacetate} \longrightarrow \text{citrate} + \text{CoASH}$$

$$\text{malate} + \text{NAD} \rightleftharpoons \text{oxalacetate} + NADH_2$$

The presence of ALC causes final NAD reduction by removing oxalacetate, and hence displacing the equilibrium of the last reaction. The method has good specificity, as the citrate synthase reaction is strictly specific for acetyl-S-CoA, but it is sensitive only in the millimolar range [2,19–21].

2.2. Chromatographic techniques

2.2.1. High-performance liquid chromatography

The use of a Supelcosil NH$_2$ (5 μm) column (250 mm$\times$4.6 mm I.D.) and UV detection at 205 nm allows the separation and quantitation of LC, ALC, PLC, the chloride ion (which often salifies the ionic group) and their likely impurities, such as acetic acid, propionic acid, crotonoyl betaine and crotonoyl betainamide, as shown in Fig. 1. However, this assay is not enantioselective and has very low sensitivity, although it has good specificity and thus is specifically used in raw material and pharmaceutical formulation monitoring (Table 1). With the NH$_2$ column, elution is inversely related to the length of the acyl group. This correlation is inverted when using a reversed-phase column. Derivatization of LC as 4'-bromophenacyl ester as proposed by Minkler and
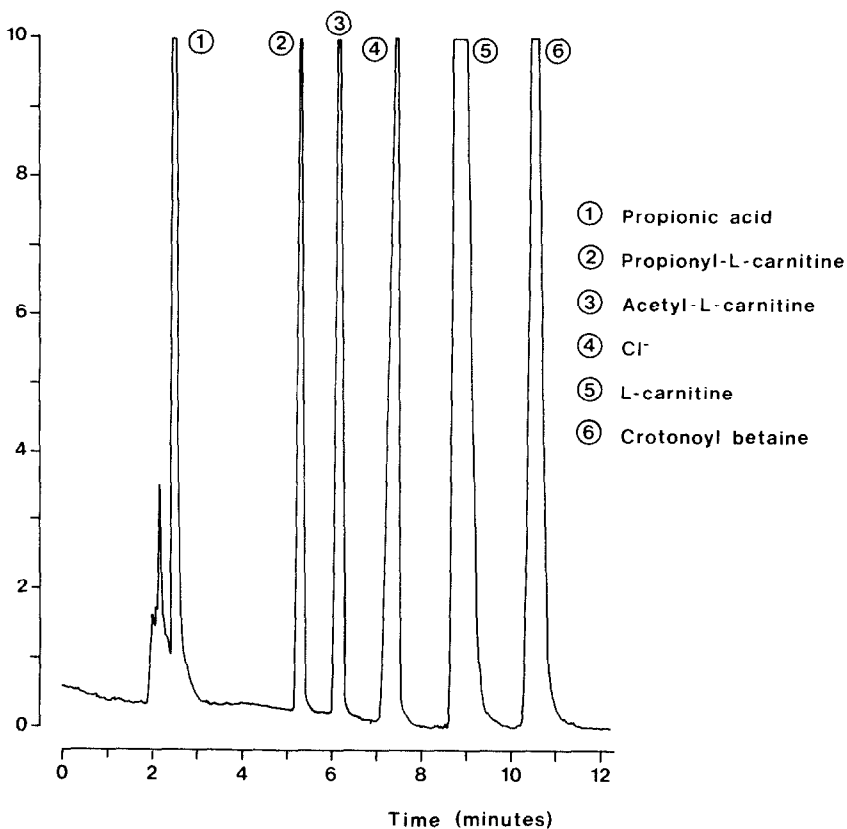

Fig. 1. Typical HPLC recording showing the separation of L-carnitine, some L-carnitine esters, some likely impurities and chloride ion. A Supelcosil NH$_2$ (5 μm) column (250 mm$\times$4.6 mm I.D.) and detection at 205 nm were used; more details are reported in ref. 40.

TABLE 1

SUMMARY OF THE MAIN CHARACTERISTICS OF ASSAYS OF THE L-CARNITINE FAMILY

Substrate	Assay	Enantio-selectivity	Absolute sensitivity (mol)	Application	Reproducibility (C.V., %)	Refs.
LC + individual esters	HPLC	No	10^{-7}	Pharmacy Urine analysis	1 7	40 22, 23
LC + individual esters	HPLC–enzyme	Yes	10^{-7}	Urine and tissue analysis		24, 25
LC	GC	No	10^{-7}	None		26
Acyl component typization	GC	No	10^{-7}	Urine analysis		41–43
LC individual esters	NMR	Yes	10^{-5}	Pharmacy		27
LC, ALC SCLC	Spectrophoto-metric–enzyme	Yes	10^{-8}	Pharmacy and urine analysis	3.7	12, 15, 16
LC, ALC	Radioenzyme	Yes	10^{-12}	Pharmacokinetics	1.7–4.8	28, 30, 31, 35
LC, SCLC	Radioisotopic exchange	Yes	10^{-12}	Pharmacokinetics	0.5–14.7	37–39

co-workers [22,23] allows LC to be evaluated with UV detection at 254 nm with increased sensitivity that is enough for urine (but not for plasma) evaluation. This method is not enantioselective either.

The use of CAT promotes the enzyme reaction in which the formation of acyl-S-CoA is stoichiometrically related to acyl-L-carnitine. The evaluation of CoA esters gives a good enantioselective evaluation of LC which is sensitive enough for measurement in some tissues and in urine (but again not in plasma) [24,25]: a C_8 Zorbax ($5\,\mu$m) column ($250\,\text{mm} \times 6.0\,\text{mm}$ I.D.) and UV detection at 254 nm are employed.

High-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC), associated with a CAT-mediated radio exchange, can provide

very sensitive evaluation of the relative distribution of LC and its esters, as mentioned in Section 2.4.3.

2.2.2. Gas chromatography

The ionic and polar nature of LC renders this technique meaningless for any analytical purpose, at least on the drugs themselves. These molecules are unstable at temperatures above $100\,^\circ$C and break up, giving rise inter alia to crotonoyl betaine, unsaturated butyrolactone and trimethylamine. A gas chromatographic (GC) method has been described [26] for the determination of LC, based on its decomposition at $160\,^\circ$C in the presence of NaOH and NaBH$_4$. The decomposition product has been identified as 4-butyrolactone. As discussed in Section 3, GC should be considered to be the technique of choice for analyses of the fatty acid component of LC esters after specific extraction, which involves alkaline hydrolysis and esterification of the fatty acids.

2.3. Spectroscopic evaluations

2.3.1. NMR technique

Nuclear magnetic resonance spectroscopy with chiral shift reagents allows LC and ALC to be quantitatively and enantioselectively evaluated [27]. The method was applied by us to PLC, using [Eu(hfc)$_3$] as chiral shift reagent. The addition of such chiral additive in a 1:1 ratio distinguishes very clearly between L- and D-enantiomers, which can be easily integrated (Fig. 2). This method works in the millimolar range, and thus it is employed in characterizing raw material of LC and its esters.

2.4. Towards a highly efficient radioenzyme assay

The pharmacokinetics of components of the LC family are now generally investigated using highly sensitive reproducible enantioselective radioenzyme assays. An assay allows the evaluation of free LC and "total carnitine", the sum of free and esterified LC after alkaline hydrolysis. Another direct assay is routinely available for ALC. The problem of analysing short-chain LC esters (C$_2$–C$_5$) is approached by a CAT-mediated radioexchange method. All these methods are extensively reviewed in the following sections.

2.4.1. L-Carnitine

A significant improvement in the sensitivity of the LC assay was achieved by Cederblad and Lindstedt [28], who used [1-^{14}C]acetyl-S-CoA, which yields labelled ALC in the presence of CAT, allowing LC to be determined at the picomole level. ALC was separated from unreacted labelled acetyl-S-CoA by passing the reaction mixture through an anion-exchange resin column.

The method is free from interference owing to the presence of SH groups in

Propionyl-L-carnitine (PLC) 4.7 mg
Propionyl-D-carnitine (PDC) 2.8 mg
in 6 ml of CD$_3$OD
+ Eu(hfc)$_3$ 39 mg

PLC

PDC

Propionyl-L-carnitine 4.7 mg
Propionyl-D-carnitine 2.8 mg
in 6 ml of CD$_3$OD

PLC
+
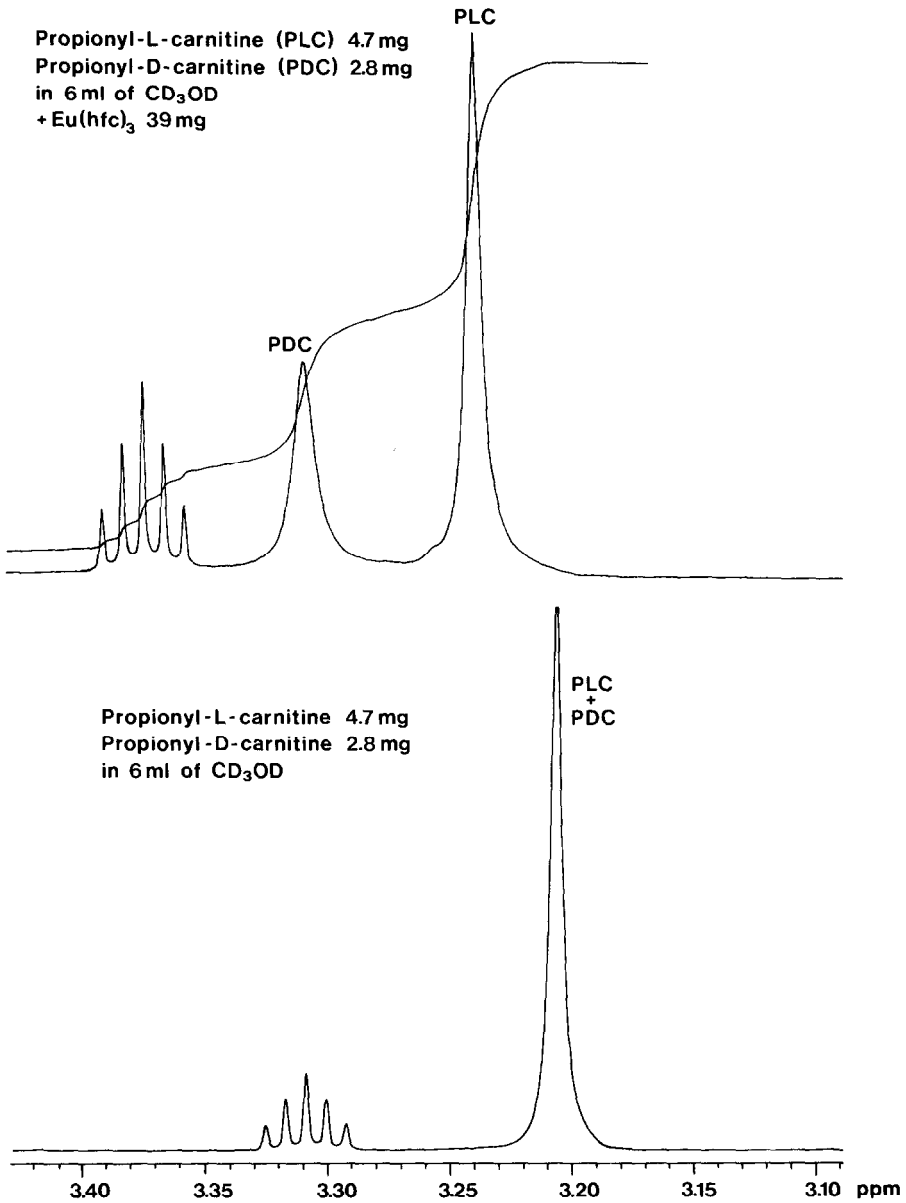PDC

3.40   3.35   3.30   3.25   3.20   3.15   3.10 ppm

Fig. 2. Typical NMR spectra of a mixture of propionyl-L-carnitine and propionyl-D-carnitine in the presence and in the absence of Eu(hfc)$_3$. Unfavourable sensitivity confines this method to only raw material analysis.

the sample and it has the advantage of high sensitivity. However, because of the low equilibrium constant of the reaction, an excess of labelled acetyl-S-CoA is required to obtain linear standard curves. Moreover, significant errors arise from the presence of unlabelled (endogenous) ALC in the assay mixture, as it takes part the reversible equilibrium. To obtain a better relationship between LC to be assayed and ALC formed, Bohmer et al. [29] used DTNB to displace the equilibrium to the right by trapping the CoASH released, but this reagent was found to inhibit CAT and also to suffer from interference from thiol compounds.

A more valuable modification of the Cederblad procedure was proposed by McGarry and Foster [30], who substituted DTNB with sodium tetrathionate. In this way they obtained linear standard curves, even in the presence of large amounts of ALC. LC determined without any treatment was the "free LC" and that calculated after hydrolysis with KOH (which comprises free plus esterified LC) was called "total LC". Moreover, increased rapidity and simplicity were achieved by adding the anion-exchange resin to the test-tube and, as regards plasma, measuring free and total LC in samples without extraction.

In a further modification, Parvin and Pande [31] demonstrated that N-ethylmaleimide (NEM) is more efficient in preventing a reversal of the CAT reaction because it removes CoASH more rapidly than tetrathionate. Tetrathionate was also observed to inhibit CAT. Any interference due to the presence of ALC can be overcome by including NEM in the assay. This is important in some pathological cases. In fact, physiological ALC levels are not so high as to disturb the LC assay, but the plasma level of ALC may be three times the free LC level in some cases, e.g. samples from subjects with renal failure. A minor modification was the use of charcoal, instead of an anion-exchange resin, to separate ALC from acetyl-S-CoA and to purify the tissue extract before the free LC assay.

Further differences between the methods described involve the extraction procedure for the sample, which is particularly important for tissue determination, and the other reagents used, mostly buffers. In this regard, the perchloric acid method has been found to be the most suitable for preparing tissue extracts [32], and many buffers can be used (except Tris, which has been found to cause non-enzymic acetylation [33]).

The perchloric acid extraction allows a partition between a "total acid-soluble" fraction, present in the supernatant, comprising free LC and its short-chain esters, and an "acid-insoluble" fraction, present in the pellet and comprising long-chain LC esters. Medium-chain acyl-L-carnitine has a critical solubility, so that it is difficult to standardize its extraction.

Moreover, particular care should be taken in free LC determination when the presence of significant amounts of endogenous acetyl-S-CoA is suspected, as this will dilute radiolabelled acetyl-S-CoA. In this case, the passage of the perchloric acid extract through an anion-exchange resin or a higher concen-

tration of the radioactive compound would eliminate this interference [27,34]. There is no problem here in the total LC assay since, as with alkaline hydrolysis, any endogenous acetyl-S-CoA will also be hydrolysed. LC is stable over a wide pH range, but acyl-L-carnitine is rapidly hydrolysed in alkaline solutions. Moreover, in vivo, ALC is easily equilibrated with free L-carnitine through the CAT catalysis.

All these substances are markedly affected by bacterial action. Attention must therefore be paid to the storage of samples, in order to avoid invalidating the assay because of changes in the amount and pattern of LC. Tissues must be immediately frozen in liquid nitrogen and then rapidly homogenized and deproteinized or stored in liquid nitrogen or at $-80\,^\circ$C, otherwise hydrolysis of acyl-L-carnitine occurs. Plasma is stable for several months at $-20\,^\circ$C. In urine, LC esters are destroyed by microorganisms, rather than by chemical or enzymic reactions. It is therefore advisable, particularly when collection is carried out over prolonged times, to collect urine in the presence of an antibacterial agent, such as NaN_3 (1 g/l) or thymol [5 ml of a 10% (w/v) solution in 2-propanol per litre of urine]. Urine should be refrigerated during collection and then frozen immediately at $-20\,^\circ$C.
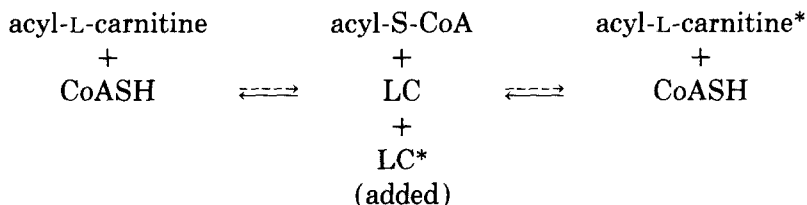
2.4.2. Acetyl-L-carnitine

A sensitive radioisotopic procedure, which enables ALC to be estimated at the picomole level, was proposed in 1980 by Pande and Caramancion [35]. As described, earlier acetyl-S-CoA is first formed from ALC in the reaction catalysed by CAT. In the presence of an excess of [U-^{14}C]oxalacetate, acetyl-S-CoA is then converted into [^{14}C]citrate. After conversion into aspartate catalysed by glutamine oxaloacetic transaminase (GOT), unreacted oxalacetate is trapped by a cation-exchangé resin, while the radioactivity of the citrate formed is measured. Its good specificity and excellent sensitivity make this method reliable for estimating ALC in perchloric acid extracts of tissues and biological fluids. Free LC, tested up to 5000-fold molar excess, was not shown to invalidate the assay. Interferences from propionyl-S-CoA, PLC and other short-chain LC esters are possible, but only if present in very large amounts compared with ALC. Endogenous acetyl-S-CoA, oxalacetate and citrate of course participate in the system, but their interference (when suspected) can be avoided by including a purification step, with an anion-exchange resin, prior to the assay. A modified procedure specifically designed to avoid citrate interference has been proposed by Cooper et al. [36].

2.4.3. Propionyl-L-carnitine and acyl esters (C_2–C_5)

Like other short-chain LC esters (other than ALC) PLC does not have an individual radioenzyme assay, as no specific biochemical reactants are routinely available. The most reliable and sensitive procedure is currently a ra-

dioisotopic exchange method described by Kenner and Bieber [37,38] and Bieber et al. [39], which enables picomole amounts of short-chain acyl-L-carnitine up to five carbon atoms to be identified and measured. An aliquot of the "total acid-soluble" fraction, containing a pool of free LC and short-chain acyl-L-carnitine, is incubated with labelled LC in the presence of CoASH and CAT As the reaction catalysed by CAT is easily reversible, an isotopic equilibrium is attained, according to the following scheme:

$$
\begin{array}{ccccc}
\text{acyl-L-carnitine} & & \text{acyl-S-CoA} & & \text{acyl-L-carnitine*} \\
+ & & + & & + \\
\text{CoASH} & \rightleftharpoons & \text{LC} & \rightleftharpoons & \text{CoASH} \\
& & + & & \\
& & \text{LC*} & & \\
& & \text{(added)} & &
\end{array}
$$

Radioactive acyl-L-carnitine is then separated by either HPLC or TLC, and the radioactivity of each fraction is counted. Amounts of acyl-L-carnitine in individual fractions are calculated by comparing the radioactivity of each fraction with that of the known amount of the initial total LC (or free LC) pool, both determined by the radioenzyme assay. This method is very simple and sensitive. It has been applied successfully to different biological fluids and tissues.

However, problems have been encountered with some urine samples. These may, at least in part, be due to the typical pattern of LC present in urine as a consequence of the substance acting as a carboxylate scavenger.

As recently stated by Marzo et al. [40] for PLC, an acyl ester of L-carnitine can be chemically hydrolysed to LC, which is then evaluated using the enzyme assay [12] as an expression of the given acyl ester.

2.4.4. Long-chain acyl-L-carnitine

As these L-carnitine esters are insoluble in perchloric acid, they can be measured in the precipitate obtained from perchloric acid extraction, after alkaline hydrolysis according to the method described in Section 2.4.1 for free LC. However, we found that current procedures are inadequate as recovery is poor, because of the perchloric acid treatment and may be the subsequent neutralization step as well.

3. CHARACTERIZING L-CARNITINE ESTERS

LC interacts easily in the body with carboxylates activated as acyl-S-CoA in the presence of specific transferases. From this viewpoint LC esters possess better renal clearance than either LC or carboxylic acid. In fact LC esters account for ca. 15% of total carnitine in plasma and ca. 50% in the urine. Examples of this conjugation include valproic acid and pivalic acid, which are excreted via the urine as LC esters [41]. This calls for LC esters in urine to be

characterized, particularly in given pathological situations [42,43]. The most common approach to this is to extract the urinary LC esters. This procedure requires free fatty acids and neutral lipids to be eliminated by solvent partition. The remaining water fraction is then chemically hydrolysed, and the carboxylic acid products are extracted and analysed. This can be carried out, after esterification of the carboxylate, by GC and detection with flame ionization or coupled mass spectrometry (MS) [42,43]. These esters have in some cases been directly identified by fast atom bombardment MS [44–46].

4. SUMMARY

L-Carnitine and its acyl esters constitute an endogenous pool of the L-carnitine family, involved in the uptake of free fatty acids in the mitochondria by transfer across their membrane of the acyl moieties to fuel the β-oxidation and the release of the acetyl group from the mitochondria to the cytosol. Therefore acyl-L-carnitine and acyl-L-carnitine transferase are involved in a homeostatic equilibrium with the cells. As most of these substances need to be monitored in foods, chemical and pharmaceutical processes and biological fluids, an overview of the main methods for assaying them is provided here, with specific reference to the intrinsic performance of each analytical procedure and with suggestions on the correct storage and manipulation of analytical samples.

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